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Strand Replacement of Plasmid R1162 and Transport of MobA During Conjugative Transfer

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Dedication

This is dedicated to my family for their unending encouragement and help.

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Strand Replacement of Plasmid R1162 and Transport of MobA During Conjugative Transfer

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R1162 is a broad-host range, mobilizable plasmid conferring resistance to streptomycin and sulfonamides. Efficient conjugative mobilization of R1162 requires three plasmid-encoded proteins: MobA, MobB and MobC. MobA binds plasmid DNA at the origin of transfer (*oriT*), nicks the subsequently transferred strand and ligates the ends of the strand after transfer into the recipient. The N-terminal region of this protein carries out this DNA processing. The C-terminal half is a primase required to initiate DNA synthesis at two single-stranded priming sites sites, *oriL* and *oriR*, during vegetative plasmid replication. The primase region of MobA is not necessary for DNA processing by the N-terminal part of the protein, however its role in strand replacement during conjugation is not clearly defined. This study demonstrates that R1162 can undergo multiple rounds of transfer from a single plasmid molecule. The presence of *oriL* increases the frequency of second-round transfer, presumably due to initiation of

replacement strand synthesis at this site by R1162 primase in the donor. Priming at *oriR* by the primase region of MobA is required for efficient replacement strand synthesis in the recipient when the plasmid is transferred to *Salmonella*. When the plasmid is transferred into *E. coli*, the plasmid-encoded priming system is not required for strand replacement in the recipient, presumably due to a host-encoded mechanism capable of priming the transferred strand. Transport of MobA through the R751 conjugative pore was also investigated. The two domains of MobA can be transported to recipient cells independently of each other. However, MobB is required for the transport of either fragment. Two sites, named the R-site and the P-site, are located in the relaxase and primase domains of MobA, respectively, and make up part of the signals required for MobA transport. Unlike previously described type IV transport signals, domain structure is required for the MobA transport signals to be active.

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Chapter 1: Introduction

1.1 PLASMID DNA REPLICATION

The struggle between development of novel antibiotics and emergence of antibiotic resistance in bacteria has been ongoing since the use of antibiotics became commonplace. Progress made by scientists and pharmaceutical companies studying new antibiotics is continually held in check by the ability of bacterial populations to become rapidly resistant to these compounds. Plasmid transfer by conjugation is one mechanism responsible for the spread of antibiotic resistance genes among bacteria (Mazel and Davies 1999). This process has been studied for many years, but there are still unanswered questions. For example, how are missing strands replaced after transfer of a single strand of DNA to the recipient cell? What roles do the proteins used during plasmid transfer play in targeting the plasmid to the transfer pore? Many of the mechanisms employed by plasmid R1162 during replication and conjugation have been studied in depth, making this plasmid a good model for answering these questions. To begin, however, we must first examine the processes of plasmid DNA replication and conjugative transfer.

Replication of plasmid DNA relies, to some extent, on proteins encoded by the chromosome. Despite this, initiation of plasmid replication is controlled by mechanisms other then those used to initiate chromosome replication. Replication of chromosomal DNA is initiated at a unique site on the chromosome, *oriC*, but replication of plasmid DNA is initiated at a different site on the plasmid, often termed the *oriV*. Both are initiated through the actions of various proteins, such as iteron-binding proteins, helicases and primases, but the proteins that are used can vary. The degree of reliance of a plasmid on host-encoded proteins for activation of the *oriV* can affect its host-range.

Plasmids that require fewer host-encoded proteins for initiation of replication generally have a broader host-range. Once replication is initiated in these broad-host range plasmids, polymerases and other proteins provided by the host complete DNA replication.

There are various strategies utilized by plasmids for vegetative replication, but in gram-negative cells many of these strategies contain similar steps. In general, these steps include distortion of the helix, helicase loading, priming, polymerization, and ligation (del Solar et al. 1998). Many plasmid origins utilize some form of DNA-binding protein to initiate helical distortion, the first step in replication. Like DnaA in chromosome replication, these proteins bind directly repeated sequences (iterons) in the origin of replication and locally distort the DNA helix to allow loading of a helicase. The helicase then unwinds the DNA to allow priming of each strand. Some plasmids use DnaA, either alone or with the aid of other DNA binding proteins, to distort the helix. In the case of plasmid pSC101, DnaA binds specific sequences within the origin of replication (DnaA-boxes) while a plasmid-encoded protein, RepA, binds a separate set of iterons (Vocke and Bastia 1983; Stenzel, MacAllister, and Bastia 1991). Interaction between these proteins, bound at their respective iterons, distorts the DNA helix at *oriV* to form an open complex (Sharma, Kachroo, and Bastia 2001). Integration host factor (IHF) assists in the interaction between DnaA and RepA by binding a site located between the RepA and DnaA binding sites and bending the helix so that bound DnaA and RepA are brought close together (Stenzel, MacAllister, and Bastia 1991). After formation of the open complex, RepA binds DnaB and loads this helicase onto the separated strands (Datta, Khatri, and Bastia 1999). DnaA must be in an active, ATP-bound state to initiate chromosomal replication, and the concentration of available ATP-bound DnaA is tightly linked to the cell cycle (Kurokawa et al. 1999). However, *in vivo*

studies have indicated that a mutated DnaA, unable to bind ATP, can initiate pSC101 replication (Sharma, Kachroo, and Bastia 2001) and thus pSC101 replication, unlike replication of the chromosome, is not restricted to certain periods within the cell cycle.

DnaA also plays a role in the replication of plasmid RK2 in *E. coli*. The plasmid-encoded initiation protein, TrfA, is translated from two, in-frame start sites, resulting in two forms of the protein, named TrfA-33 and TrfA-44 (Shingler and Thomas 1984). Either form of TrfA can bind to the iterons in the RK2 origin of replication and, with the help of HU protein, distort the helix at an AT-rich region within the origin (Konieczny et al. 1997). DnaA binds DnaA-boxes adjacent to the iterons and is capable of stabilizing this open complex, but cannot, by itself, distort the DNA at the AT-rich region. In *E. coli*, DnaA is required to recruit DnaB helicase to the plasmid origin of replication, but TrfA is required for helicase activity, presumably because only TrfA can distort the helix to allow loading of the helicase (Konieczny and Helinski 1997). However, in *Pseudomonas putida* and *Pseudomonas aeruginosa* the DnaB helicase is loaded and activated solely by the TrfA-44 form of the initiator, and is thus a DnaA-independent mechanism for initiation of RK2 replication (Jiang et al. 2003).

Other plasmids, such as R1162 (Figure 1.1A; Barth and Grinter 1974), do not use DnaA protein at all, but rely entirely on their own iteron-binding protein. Originally isolated from *Pseudomonas*, R1162 is a member of the IncQ family of plasmids and is virtually identical to the plasmid RSF1010 from *E. coli* (Guerry, van Embden, and Falkow 1974) and R300B from *Salmonella typhimurium* (Grinter and Barth 1976). Plasmids of the IncQ family are replicated from a single locus, *oriV* (Figure 1.1B), which consists of three and a half iterons, a GC-rich region, an AT-rich region, and two, oppositely-oriented priming sites, *oriL* and *oriR* (Lin and Meyer 1987; Scholz et al. 1989). Using purified protein, DnaA is not required for initiation of plasmid replication

at *oriV in vitro* (Scherzinger et al. 1991). The replication of R1162 is instead initiated by a plasmid-encoded, iteron-binding protein, RepC. This protein binds to the *oriV* iterons, distorting the DNA helix at the AT-rich region to form an open complex (Kim and Meyer 1991).

A plasmid-encoded, hexameric helicase, RepA, unwinds the DNA from the AT-rich region by translocation in a 5' to 3' direction (Scherzinger et al. 1997). The helicase activity of RepA exposes the single-stranded initiation sites, *oriL* and *oriR*, which are then primed by the plasmid-encoded primase (Haring and Scherzinger 1989). Unlike the *E. coli* primase, DnaG, which produces a RNA primer, RepB' synthesizes a primer composed of both ribonucleotides and deoxyribonucleotides.

The R1162 primase is expressed in two forms: as a fusion with the R1162 relaxase, MobA, with the primase the C-terminal end of the protein, and as a shorter, primase-only form created by translation from an alternate, in-frame start site (Scholz et al. 1989). Both forms of the primase are functional, and to date the *oriV* priming sites are the only known sites recognized by each.

The two priming sites are interchangeable and replication of either strand can be initiated independently (Zhou and Meyer 1990). Unlike chromosome replication, there is no lagging strand replication (Scherzinger et al. 1991). Therefore the nascent DNA strands displace the parent strands, resulting in single stranded D-loops extending in both directions until the replication forks pass one another. It was shown in an earlier study that loss of either priming site does not prevent replication of the plasmid (Zhou and Meyer 1990). However, compensation for the loss of *oriL* appears to be provided by an SOS induced, host-encoded system (Becker, Zhou, and Meyer 1996).

1.2 CONJUGATIVE TRANSFER OF PLASMID DNA

Conjugation is a process by which genetic information, commonly in the form of plasmids, can be transferred within bacterial populations through cell-to-cell contact (Watson and Hayes 1953). During bacterial conjugation, a cell harboring a transmissible plasmid forms a physical connection with another cell. The second cell then receives a copy of the plasmid DNA from the donor cell so that both cells now contain the plasmid. The connection is then broken and the bacteria can repeat the process with other nearby cells. In this manner, a transmissible plasmid can be spread quickly throughout a population of bacteria.

Transmissible plasmids encode the proteins required to perform two distinct steps of conjugation. The first of these, DNA processing, is accomplished with relatively few proteins. Transmissible plasmids encode a protein called the relaxase, which binds at the origin of transfer (*oriT*) and, along with various accessory proteins, forms a nucleoprotein complex termed the relaxosome (Furste et al. 1989; Lanka and Wilkins 1995). Within the relaxosome, the strands of duplex *oriT* DNA are partially separated, allowing the relaxase to nick one of the strands by transesterification, resulting in tyrosyl phosphodiester linkage of the relaxase to the 5' end of the cleaved strand (Lanka and Wilkins 1995; Byrd and Matson 1997). The strand linked to the relaxase is then transferred to the recipient cell. After transfer of the plasmid strand, the covalently-linked relaxase rejoins the ends of the transferred, single-stranded molecule by reversal of the transesterification reaction and release of free protein (Byrd and Matson 1997).

The second step of conjugation is the translocation of DNA from one cell to another. Unlike DNA processing, this second step occurs by a more complicated mechanism. Transmissible plasmids, such as F, RP4 and R751, transfer DNA through a type IV translocation complex similar to the VirB/D4 machinery used by *Agrobacterium*

tumifaciens to deliver tumeragenic DNA (T-DNA) to plant cells. This complex consists of 8-11 proteins (Frost, Ippen-Ihler, and Skurray 1994; Thorsted et al. 1998), but the functions of many of these proteins are still unclear. There are differences between the conjugative systems encoded by unrelated plasmids, but the homology of the encoded proteins suggest that the basic architecture of the translocation machinery is similar (Schroder and Lanka 2005). The pilus, made up of pilin subunits, connects the donor and recipient cells in a process called mating pair formation (Mpf). The structure of the machinery responsible for translocation of DNA through the transfer pore and the functions of the components making up this pore remain unclear. Three proteins are ATPases thought to provide the energy necessary to transfer the DNA through the pore, but the actual mechanism driven by this energy is still unknown (Christie and Vogel 2000). Other components of the transfer machinery appear to form a bridge between the inner and outer membranes to allow transfer of substrates through the periplasmic space. Crosslinking of T-DNA to pore proteins as it passed through the *Agrobacterium* T-pilus showed that the T-DNA makes physical contact with at least five of the transfer machinery proteins in a sequential manner (Cascales and Christie 2004).

Common among type IV transfer systems is the coupling protein, which links the transported substrate to the transfer machinery and is essential for transfer (Disque-Kochem and Dreiseikelmann 1997; Hamilton et al. 2000; Schroder et al. 2002; Cabezon, Sastre, and de la Cruz 1997). During transfer of plasmid DNA, the coupling protein interacts with the relaxase (Szpirer, Faelen, and Couturier 2000), which is then delivered to the transfer pore (Cascales and Christie 2004). In one case, the coupling protein forms a hexameric ring associated with the cytoplasmic membrane but it is still unknown whether any transferred substrate actually passes through the lumen of this ring (Gomis-Ruth et al. 2001). The coupling protein is also one of the three pore components that

possess ATPase activity. Although this activity is not required for binding to relaxases or DNA *in vitro*, it is required for transfer (Schroder and Lanka 2003; Schroder et al. 2002).

In addition to their role in DNA transfer, type IV systems also transport into eukaryotic cells proteins unlinked to DNA. For example, the VirB/D4 system of *Agrobacterium* transfers proteins VirE2, VirE3, and VirF in addition to nucleoptrotein (T-DNA). The Dot/Icm system of *Legionella* mobilizes protein RalF, LidA, and DotA (Christie et al. 2005). Although this system is normally used for transport of effector proteins into eukaryotic cells, it is also able to mobilize plasmid RSF1010 into *Legionella* and *E. coli* recipients (Vogel et al. 1998). As with the relaxase molecules, effector proteins also interact with the coupling protein prior to transfer (Llosa, Zunzunegui, and de la Cruz 2003). There is a grouping of positively charged amino acids, often in the configuration R-X-R, approximately 10-15 amino acids from the C-terminal end of some of these effector proteins. Loss of these amino acid residues prevents transfer through the pore (Luo and Isberg 2004; Vergunst et al. 2005). However, recent data also suggest that there may be other requirements for transfer (Hohlfeld et al. 2006; Schulein et al. 2005). The nature of the interaction of transported substrates with the coupling protein has not yet been determined. However, it has been suggested that the C-terminal end of the transferred protein, containing the positively charged residues, is unstructured and that the coupling protein recognizes this C-terminal "tail" (Nagai et al. 2005).

Unlike transmissible plasmids, mobilizable plasmids encode the proteins needed for DNA processing but not those needed to form a transfer pore. Thus, they depend on transmissible plasmids to supply the type IV transfer machinery. R1162 is a mobilizable plasmid that efficiently utilizes the type IV system of R751 and other transmissible plasmids of the IncP group (Meyer, Hinds, and Brasch 1982). It encodes

three proteins involved in relaxosome formation: MobA, MobB and MobC (Figure 1.1A) (Derbyshire, Hatfull, and Willetts 1987). MobA, the relaxase, is the largest of these proteins (Derbyshire, Hatfull, and Willetts 1987). The *oriT* recognized by this relaxase consists of 37 base pairs and is composed of three regions: an inverted repeat, an AT-rich region, and a unique nicking site (*nic*) (Brasch and Meyer 1987). After MobA binds *oriT*, the AT-rich region of the DNA is locally melted and a specific strand within the unpaired region is cleaved at *nic* (Scherzinger et al. 1992). MobC enhances strand separation at *oriT*, thereby enhancing nicking by allowing MobA better access to single-stranded DNA at *nic* (Zhang and Meyer 1997). MobB stabilizes the binding of MobA to duplex *oriT* DNA (Perwez and Meyer 1996). This stabilization increases the number of molecules prepared for transfer, thereby increasing the number of transconjugants per donor. However, the stabilization of the relaxosome and enhancement of transfer could be separated by mutation (Perwez and Meyer 1999). This suggests that there may be another role for MobB in transfer.

TraG, the coupling protein encoded by the transmissible helper plasmid R751, presumably targets MobA-linked DNA to the transfer pore. There is no direct evidence of this for R1162 but interaction between the relaxase and TraG has been shown with the mobilizable plasmid pBBR1 (Szpirer, Faelen, and Couturier 2001). It is also clear that the relaxase can be passed to the recipient cell while either linked or unlinked to DNA (Luo and Isberg 2004; Draper et al. 2005). Interestingly, one study reported that transport of TrwC, the relaxase encoded by plasmid R388, is enhanced when the relaxase is linked to DNA (Draper et al. 2005). At termination of strand transfer, the cleavage reaction is reversed, resulting in a closed loop of single-stranded DNA. This reaction requires a hairpin structure formed by the inverted repeat, part of *oriT*, located at the 3' end of the transferred DNA (Bhattacharjee, Rao, and Meyer 1992). The hairpin

provides a region of double-stranded DNA for binding by MobA, thereby bringing the ends of the transferred DNA close together and allowing rejoining.

1.3 STRAND REPLACEMENT DURING CONJUGATION

Once the DNA is transferred, it must then be converted to the double-stranded form. Some plasmids, such as ColI and RP4, encode primases that are transported to the recipient cell specifically for this purpose (Chatfield et al. 1982; Merryweather, Barth, and Wilkins 1986). After transfer of R1162, the single-stranded molecule in the recipient contains one of the initiation sites for the R1162 primase. This site might be used to initiate complementary strand synthesis in the recipient (Figure 1.3). Likewise, strand replacement in the donor may be initiated at the other priming site as *oriV* becomes exposed during strand transfer. However, since R1162 is maintained at about ten copies per chromosome in the donor, it might not be necessary to rescue the remaining strand of the transferred plasmid in the donor if loss of this plasmid can be compensated for by replication of the plasmids remaining in the cell. The possibility of rolling circle replication as a mechanism for strand replacement in the donor is attractive because it suggests a way for the plasmid to be replicated as strands are transferred to the recipient. There is some evidence that strand replacement of R1162 in the donor can be accomplished by extension of the 3' end of the transferred strand, reminiscent of rolling circle replication, but the low frequency of molecules exhibiting this form of strand replacement indicate that this is not the primary mechanism used by R1162 (Erickson and Meyer 1993).

In order to utilize the single-stranded priming sites in the donor or recipient, there must be an available source of the R1162-encoded primase, RepB'. In the case of strand replacement in the donor the primase would be encoded by the other plasmids in the cell. However, the recipient cell would not contain any RepB' at the time of strand

transfer and the source of primase for strand replacement initiated at *oriV* is less obvious. As mentioned above, the MobA protein of R1162 is unusual in that its C-terminal end consists of the RepB' primase. This fusion of relaxase and primase might be responsible for *oriV*-driven strand replacement in the recipient since the *oriV* is near *oriT*. Once MobA is linked to *oriT* it would be close to *oriV*, allowing MobA a chance to initiate strand replacement at the exposed priming site. Under conditions of low-frequency mobilization, where efficient strand replacement in the recipient is more important for establishment of the plasmid, formation of transconjugants required primase linked to the relaxase (Henderson and Meyer 1996). Furthermore, supplying the short form of the primase in the recipient cell did not relieve this requirement (Henderson and Meyer 1999). These observations suggest that linkage of the primase to MobA can be involved in establishment of R1162 following transfer. However, this fusion between relaxase and primase is not a feature of all mobilizable plasmids, and therefore is not strictly required for strand replacement. Plasmids utilizing host-encoded mechanisms would not necessarily need to transport plasmid-encoded replication factors into the recipient cell. In fact, the primase region of the R1162 relaxase can be removed without loss of transfer, as long as another mechanism for initiating strand synthesis, such as the ColE1 replicon, is supplied (Brasch and Meyer 1986).

Aside from its possible role in strand replacement, what other function might the MobA-linked primase perform? In many of the type IV secretion systems studied to date, the transfer signal of proteins targeted for transport are located, at least in part, within the C-terminal end (Schulein et al. 2005; Hohlfeld et al. 2006; Nagai et al. 2005; Luo and Isberg 2004; Vergunst et al. 2005). One recent study showed that the C-terminal 48 amino acids of RSF1010 MobA are sufficient to target a protein for transfer through the *Agrobacterium* Vir pore (Vergunst et al. 2005). In light of these studies,

conjugative transfer of the R1162 nucleoprotein complex in the absence of the C-terminal primase region is surprising. Removing the primase region should result in loss of the transfer signal and failure to transfer. Thus, either MobA contains multiple transfer signals, or different signals are used for the Vir and IncP pores. The purpose of this study was first to first determine the role of MobA in conjugative strand replacement, and then to define possible transfer signals responsible for selection of the nucleoprotein complex as a substrate for transport.

Chapter 2. Materials and Methods

2.1 STRAINS AND PLASMIDS

Strains used in this study are listed in Table 2.1. Plasmids used to study strand replacement during conjugation are listed in Tables 2.2 while those used to study transport of MobA are listed in Table 2.3. Oligonucleotides used in the construction of plasmids and strains are listed in Table 2.4.

Test plasmids possessing both priming sites (*oriL* and *oriR*) but lacking the genes required for replication (Figure 2.1) were maintained as satellite plasmids in strains containing a helper plasmid, pMS94, which supplied the replication proteins. Test plasmids lacking one, or both, of the priming sites were maintained as a fusion with plasmid pBR322. These chimeric plasmids were created by ligating BamHI-digested pBR322 into the BamHI site of the test plasmid.

The R751 (*traC*$^-$) plasmid (pUT1757) was constructed by first flanking the non-polar, promotorless kanamycin resistance gene in pUC18K with fragments of *traC,* each approximately 500 base pairs in size and complementary to bases pairs 39449-39942 and 41584-42091 of R751 (Thorsted et al. 1998). The fragments were obtained by PCR amplification of R751 DNA with primers 388 and 389, and 386 and 387, and were ligated into pUC18K after digestion with BamHI and PstI, and EcoRI and KpnI, respectively. The resulting plasmid was then introduced into a strain harboring naturally occurring R751. Homologous recombination of the kanamycin resistance gene into the *traC* locus was detected by screening for kanamycin-resistant, ampicillin-sensitive transconjugants following transfer of the altered R751 plasmid into a new host cell.

The Top10::LKL strain was constructed by first creating a Cre sensor located on a plasmid. A small duplex oligonucleotide containing a *lox* site was created by annealing oligonucleotides 580 and 581. This DNA was cloned between the KpnI and BamHI sites of pUC19 (Yanisch-Perron, Vieira, and Messing 1985). A BamHI-KpnI fragment containing a promoterless, non-polar kanamycin resistance gene from pUC18K (Menard, Sansonetti, and Parsot 1993), and an additional duplex *lox* oligonucleotide, were both ligated into the pUC19-*lox* plasmid, resulting in a kanamycin resistance gene flanked by *lox* sites, and located in the polylinker region of pUC19. This sensor, which includes the adjacent *lacZα* gene, was amplified by primers 630 and 631. The 5' ends of these oligonucleotides are homologous to basepairs 312242-312281 and 313301-313340 of the published *E. coli* K-12 sequence (Blattner et al. 1997), respectively. The resulting PCR product was electroporated into Top10 (Invitrogen) cells harboring a temperature-sensitive plasmid, pKD46, expressing the lambda-red system (Datsenko and Wanner 2000). Integration of the Cre sensor into the chromosome was selected by kanamycin resistance and the pKD48 plasmid was cured from the strain by growing the cells at 37°C.

The Cre expression vector (pUT1885) used to prepare the Cre-MobA fusions was created by amplifying the *cre* gene of P1 with primers 611 and 652. The PCR product was cloned between the SalI and KpnI sites of pBR322. This 3'end of *cre* contained a unique KpnI site adjacent to the stop codon. In general, Cre fusions were created by amplifying the *mobA* fragment of interest by PCR such that the 5'-end of the product contained a KpnI site and 3' end contained a SalI site. This product was then cloned between the KpnI and SalI sites of pUT1885, resulting in an in-frame fusion of the *cre* and *mobA* genes. The fusions between *cre* and *mobA* were verified by submitting the expression plasmids to the Institute for Cellular and Molecular Biology core facility for sequencing.

2.2 GENERAL PROCEEDURES

Escherichia coli K-12 and *Salmonella typhimurium* LT2 strains were grown in TYE media (1% tryptone (w/v), 0.5% yeast extract (w/v), and 0.5% NaCl (w/v)). Agar was added to 1.5% (w/v) to make nutrient agar plates. When necessary, the media was supplemented with the following antibiotics: ampicillin (100 μg/mL), chloramphenicol (25 μg/mL), kanamycin (25 μg/mL), streptomycin (25 μg/mL), nalidixic acid (25 μg/mL), trimethoprim (200 μg/mL). All strains were grown at 37˚C unless otherwise indicated.

Plasmid DNA was isolated using commercially available kits from QIAgen and stored in TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) until needed. Restriction enzymes were purchased from New England Biolabs (NEB). DNA was ligated with T4 DNA ligase (NEB) in a reaction containing 1X ligation buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM dithiothreitol, 1 mM ATP, and 25 μg/mL bovine serum albumin). Oligonucleotides were purchased from Invitrogen. In cases in which the oligonucleotide was to be ligated into a plasmid, the 5'-end of the oligonucleotide was first phosphorylated with T4 polynucleotide kinase (NEB) in a reaction containing 1X ligation buffer, 500 pmol of oligonucleotide and 1 unit of kinase at 37˚C for 30 minutes.

PCR reactions contained reagents purchased from Qiagen. Reactions were in a final volume of 100 μL and contained 10 μL 10X PCR buffer (Qiagen), 50 ng of template DNA, 20 pmol of each primer, 0.2 mM dNTP and 1.5 mM $MgCl_2$. The reaction was heated to 94˚C before adding 2.5 units of Taq enzyme. For whole-cell PCR, 1 μL of overnight culture was used instead of template DNA. The general temperature profile for the reactions were 30 cycles at 94˚C for 30 seconds, 65˚C for 30 seconds and 72˚C for 1 minute, with adjustments to annealing temperature and elongation time when needed. PCR products were recovered either with QIAquick PCR

Purification Kit (Qiagen) or by isolation on a 0.8% agarose gel. The inverse PCR used in the creation of plasmid pUT1815 was performed in a similar manner except that phosphorylated oligonucleotides were used as primers and Vent polymerase (NEB) was used instead of Taq. The extension time in this reaction was increased to five minutes to allow ample time for the polymerase to proofread the PCR product during polymerization. The resulting product was gel purified and ligated to create the modified plasmid.

2.3 TRANSFORMATION AND ELECTROPORATION

$CaCl_2$ transformation: 10-mL cultures of mid-log cells were made competent by washing the cells with 5 mL of cold 0.1M $MgCl_2$, resuspending the cells in 2 mL of cold $CaCl_2$, and holding the cells on ice for 20 minutes. The cells were then pelleted and resuspended in 500 μL of cold 0.1M $CaCl_2$. Plasmid DNA was mixed with 100 μL of competent cells and held on ice for 1 hour. The cells were heat-shocked for 90 seconds at 42˚C and resuspended with 900 μL of TYE media. Cells were incubated at 37˚C for 90 minutes before being plated on selective TYE media.

Electrotransformation: 25-mL cultures of recipient cells were grown to 2 x 10^7 cells/mL and washed sequentially with 10-, 5-, and 2-mL of cold 10% glycerol. Cells were pelleted by centrifugation at 8600 x g for 10 minutes after each wash. After the last wash, the cells were resuspended in 200 μL of cold 10% glycerol. Plasmid DNA was mixed with 40 μl of competent cells and placed in an electroporation cuvette with a 0.1 cm gap width and shocked with an *E. coli* Pulser (BioRad) set at 1.8 kV. The cells were resuspended with 1 mL SOC media (2% tryptone (w/v), 0.5% yeast extract (w/v), 10 mM NaCl, 20 mM glucose, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and incubated for 90 minutes at 37˚C before being plated on selective TYE media.

2.4 STANDARD MATING ASSAYS

Overnight cultures of donor and recipient cells, grown in selective media, were diluted 10-fold in fresh TYE and grown at 37˚C for 90 minutes. Cells were then mixed together with the recipient cells in excess by a factor of at least 5. This mixture of cells was then pelleted, resuspended in 50 μL of TYE and applied directly to a TYE agar plate or drawn onto a 0.45 μM nitrocellulose filter by gentle vacuum and placed on a TYE agar plate. After incubation at 37 ˚C, the cells were resuspended in 1 mL of TYE and plated on agar plates containing the appropriate antibiotics. In the case of matings performed directly on the agar plate, the cells were resuspended by cutting out a plug of agar containing the cells and placing it in a culture tube containing 1 mL of TYE.

Cross-streak matings were performed by applying donor and recipient cells directly to TYE agar as two perpendicular lines using a loop or toothpicks. The cells were allowed to grow overnight or until growth was visible (6-8 hours). Samples were taken from the intersection of the two lines of cells and streaked onto selective media to identify transconjugants. Samples of donor and recipient cells, taken from the origin of each line, were also streaked onto selective media as a control.

2.5 ELECTROPORATION/MATING ASSAY

Supercoiled, satellite test plasmid DNA was prepared by isolating plasmid DNA from a strain containing the test plasmid and the helper plasmid pMS94, and treating this plasmid DNA with XmnI to cleave the helper plasmid. Test plasmids containing mismatched nucleotides (pUT1557 and pUT1740; Figure 2.1) were prepared by digesting these plasmids with either BanII and MfeI or ApaI and NgoMIV, respectively, and ligating into these sites heteroduplex oligonucleotides at a 1:5 (vector:insert) ratio. The NheI/FspI heteroduplex oligonucleotide for ligation into pUT1557 was prepared by mixing equimolar amounts of phosphorylated oligonucleotides 258 and 259, boiling for 5

minutes, and cooling the solution slowly to room temperature. The oriV-/oriV+ heteroduplex oligonucleotide, cloned into the test plasmid pUT1740, was prepared similarly, by using oligonucleotides 306 and 307. Test plasmids maintained as fusions with pBR322 were prepared by digesting the chimeric plasmid with BamHI and purifying the test plasmid DNA on a gel. The linear plasmid DNA was then circularized by treatment with 0.8 units of ligase in 20-50 μL total volume.

The test plasmid DNA was introduced into donor strains by electroporation. The electroporated cells were resuspended with 1 mL of SOC media and 0.5 mL of these donors were mixed with 2.5 mL of recipient cells at a concentration of 5 x 10^8 cells per mL. This mixture of cells was then either pelleted, resuspended in 100 μL SOC and spotted onto a TYE plate, or applied to a 0.45 μM nitrocellulose filter and placed, cells up, on a TYE plate. After incubation of these cells at 37˚C for 90 minutes, they were resuspended in 1 mL of TYE and 100 μL of this mixture was plated on TYE agar containing chloramphenicol and nalidixic acid to select for transconjugants. A sample of cells was also plated on TYE containing only chloramphenicol in order to determine the number of possible donors present in the mating. All resulting colonies were counted within 24 hours of plating.

2.6 PHAGE PLAQUE ASSAY

JM103 host strain was grown overnight in 2xYT media (1.6% tryptone (w/v), 1% yeast extract (w/v), 0.5% NaCl (w/v), pH7.5). M13Δe101-based phage DNA was introduced into JM103 strains by $CaCl_2$ transformation (Section 2.3). After heat-shock, various volumes (1 μL, 10 μL, 89 μL) of the cells were added to melted 2xYT+0.6% agar, along with 100 μL of the JM103 host strain, and poured on top of TYE agar plates. In cases in which the host strain contained a helper plasmid, an appropriate antibiotic was also included in the top agar.

Phage were stored by inoculating from a plaque into 2 mL of 2xYT containing 5 μL of an overnight culture of JM103 and grown for 5-6 hours at 37˚C. The viral particles were harvested by pelleting the cells in a microfuge at 17,000 x g for 1 minute and collecting the resulting supernatant. Before storage, the supernatant was heated to 65˚C for 8 minutes.

2.7 CRE TRANSFER ASSAY

Cre donors consisted of MV10 cells containing a plasmid expressing a Cre-MobA fusion, and the R751 helper plasmid. Cells also contained a plasmid expressing MobB (pUT221) or a $MobB^-$ control plasmid (pACYC184) except when the Cre-MobA fusion itself was supplying MobB from the native open reading frame (Figure 1.1). Overnight cultures in selection media of Cre donors and the Cre-sensor recipient were diluted 10-fold in fresh media and allowed to grow for an additional 90 minutes. A mixture of donor (0.5 mL) and recipient (1 mL) cells were applied to a 0.45 μM filter by vacuum. The membranes were placed on nutrient agar, cells up, and incubated for 2 hours at 37˚C. The cells were washed from the membrane with 1 mL of TYE broth, diluted 10^{-2} and 10^{-4}, and 100 μL of these dilutions plated in duplicate on TYE agar containing streptomycin, trimethoprim and 0.006% X-gal. Blue colonies were counted on the 10^{-3} dilution plates while the total number of colonies was calculated from the 10^{-5} dillution plates. Percent recombination was determined as the percentage of transconjugant colonies that were blue. Each assay was performed at least twice.

2.8 WESTERN BLOT ANALYSIS

Overnight cultures of Cre donor strains were diluted 10-fold into TYE media and grown for 90 minutes at 37˚C. Five mL of mid-log cells were pelleted, washed with 2 mL of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3)

and resuspended in 1 mL of PBS supplemented with 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride (PMSF) and help on ice for 5 minutes. The cells were heated to 65˚C for 20 minutes and cleared by centrifugation at 20,400 x g for 15 minutes. Protein concentration was determined using the Bio-Rad Protein Assay according to the manufacturers instructions.

Twenty micrograms of total protein from each cleared lysate was precipitated by mixing the lysate with ice-cold acetone in a 1:4 ratio (lysate:acetone) and cooling the mixture to -20˚C for at least 1 hour. The proteins were pelleted by centrifugation at 15,800 x g for 10 minutes at 4˚C. The protein pellets were air dried at room temperature and resuspended in 10 μL of PBS. Five microliters of sample buffer (150 mM Tris-HCl, 6% SDS, 0.5% bromothymol blue, 30% glycerol) were added and the samples boiled for 5 minutes prior to resolving the proteins on a 4%-15% linear gradient polyacrylamide gel (Bio-Rad).

The proteins were transferred to nitrocellulose at 30V overnight at 4˚C using a Mini Trans-blot Transfer Cell (Bio-Rad). The resulting blot was blocked with 1% dry milk dissolved in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 8.0). Anti-Cre antibody (Novagen), diluted 1:10,000 in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0), was used to probe the blot for 4 hours at room temperature. The blot was washed three times with TBST for 5 minutes each and then probed with goat anti-rabbit antibody conjugated with alkaline phosphatase (AP) (Novagen), diluted 1:10,000 in TBST, for 30 minutes. After washing the blot twice with TBST and twice with TBS, it was developed using an AP development buffer (100 mM Tris-HCl pH9.5, 100 mM NaCl, 5 mM MgCl2, 0.3 mg/mL nitro blue tetrazoleum, 0.15 mg/mL 3-bromo-4-chloro-5-indolyl phosphate) (Novagen).

Chapter 3. Results: Conjugative strand replacement on R1162

3.1 MULTIPLE ROUNDS OF TRANSFER CAN BE INITIATED FROM A SINGLE PLASMID MOLECULE

Early studies with transmissible plasmids such as F showed that the transferred strand is replaced in the donor by replication (Vapnek and Rupp 1971). For these low copy number plasmids, strand replacement in the donor might be needed to minimize the loss of the plasmid in the donor. The copy-number of R1162, however, is 10-20 per cell. Because of this, the occasional loss of a few plasmid copies by conjugative transfer would have little impact on the remaining population of plasmids in the donor cell. Whereas replication is essential to restore duplex plasmid DNA in the recipient, there has been no evidence that transfer of R1162 requires strand replacement in the donor cell.

I first asked whether there was strand replacement synthesis in donor cells after transfer of R1162. To do this, I determined whether multiple rounds of transfer could be initiated from a single plasmid molecule when there was no ongoing vegetative replication of the plasmid DNA. If a plasmid can undergo strand replacement in the donor, then multiple rounds of transfer from that molecule might be detected.

An electroporation/mating technique (Henderson and Meyer 1999) was modified to study strand replacement in donors that are unable to support vegetative replication of plasmid DNA. Briefly, R1162-derived test plasmids containing *oriT*, *oriV* and a gene conferring resistance to chloramphenicol, but lacking the genes encoding the proteins required for mobilization (Mob) and replication (Rep) (Figure 1.1a), were introduced by electroporation into donor strains containing various helper plasmids supplying in *trans* the Mob and Rep proteins (Rep+ donors) or only the Mob proteins (Rep- donors).

These donors also contained the transmissible helper plasmid R751, which provided the required transfer machinery. The electroporated donor cells were then immediately mated with nalidixic acid-resistant (Nal^R) recipient cells expressing the Rep proteins (Rep+ recipients). Transconjugants were selected on TYE containing nalidixic acid and chloramphenicol.

To distinguish between first- and second-round transfer, the test plasmid pUT1557 (Figure 2.1) was modified to contain a one base pair mismatch resulting in different restriction sites on each DNA strand. This was done by annealing two complementary oligonucleotides (258 and 259) and ligating the resulting heteroduplex into the BanII and MfeI sites of pUT1557 prior to electroporation into one of the donors. The mismatch was designed so that the first DNA strand transferred from the donor would contain an NheI site, while any subsequent strands transferred from the donor would contain an FspI site (Figure 3.1A). Plasmids formed from first and subsequent rounds of transfer were identified by isolating plasmid DNA from transconjugant cells and digesting with either NheI and EcoO109I or FspI and EcoO109I. Cleaving the plasmid with EcoO109I and one of the other enzymes results in a 1000 base pair fragment easily identified on an agarose gel (Figure 3.1B). Preliminary tests indicated that mismatch repair in the donor can resolve the mismatched bases in the test plasmid, resulting in a NheI or FspI plasmid prior to conjugation. Test plasmids were therefore methylated *in vitro* to minimize mismatch repair in the donors (Pukkila et al. 1983). Methylation of the test plasmid did not affect the transformation efficiency of the donors (data not shown).

It was unclear if multiple rounds of transfer would be detected after the cells were mated for 90 minutes, the amount of time normally used in electroporation/matings, so a range of mating times was used. Test plasmid DNA containing the mismatch duplex

oligonucleotide was introduced into Rep+ or Rep- donor cells and mated for 1, 2, 3 or 4 hours with Rep+ recipient cells. To get a better representation of the population of transferred molecules, 25 transconjugants from each mating were pooled and plasmid DNA was extracted from each pool and analyzed (Figure 3.1B). Unmated recipient cells (0 hour) were used as a negative control. With a mating time as short as one hour, the transconjugant populations arising from the Rep+ donors harbored equal numbers of NheI-containing plasmids (NheI plasmids) and FspI-containing plasmids (FspI plasmids). The equal numbers reflect the vegetative replication within the Rep+ donors and conversion of mismatch-containing test plasmid into two, equally-sized sister populations of plasmids containing either NheI or FspI restriction sites. These data indicate that vegetative replication occurs soon after introduction of the test plasmid into the donor strain, within the first hour of mating. Additionally, neither NheI nor FspI plasmids have an advantage in replication, as the transconjugant populations contain similar amounts of the two forms for each time point. On the other hand, the pooled plasmid DNA samples for the matings with Rep- donors consisted of mostly NheI plasmids (Figure 3.1B). Interestingly, the ratio of NheI plasmids to FspI plasmids did not change over time, indicating that longer mating times (up to 4 hours) did not increase the chance of observing second round transfer. However, the plasmid DNA was isolated from a population of transconjugants, making it impossible to determine the distribution of FspI plasmids among individual transconjugants. Therefore, plasmid DNAs isolated from individual colonies of transconjugants resulting from the one-hour matings were analyzed (Figure 3.2A, B). Nearly half of the transconjugant colonies from matings with the Rep+ donor contained both types of plasmid, and about 30% of the transconjugants harbored only FspI plasmids. The presence of transconjugants with only FspI plasmids supports the idea that vegetative replication of the test plasmid in the donor occurs

quickly compared to transfer. The transconjugants containing both types of plasmid indicate that multiple rounds of transfer can occur, but it is unclear whether the transferred strands originated from the same plasmid molecule or from separate sister plasmids resulting from vegetative replication. We saw something different in the transconjugants arising from the Rep- donor (Figure 3.2B, line 2). In this case, the majority of the transconjugant colonies contained NheI plasmids, supporting the data from the population analysis (Figure 3.1B). FspI plasmids were found only in colonies also containing NheI plasmids. The lack of transconjugants containing only FspI plasmids is evidence that there is little mismatch repair prior to mating in these experiments.

One possible explanation for transconjugants containing both types of plasmid is that the transferred strand contained both restriction sites. Multiple copies of the heteroduplex oligonucleotide could be ligated into the plasmid, but since the two ends of the duplex molecule contain different overhangs an odd number of duplex DNAs would have to be ligated in a head-to-head, tail-to-tail orientation. Test plasmids containing three duplex oligonucleotides ligated in this fashion would result in a transferred strand containing both NheI and FspI sites. To examine this possibility, mismatch test plasmid DNA was introduced into the Rep- donor cells and plasmid DNA was collected from a pool of 25 transconjugants. This DNA was digested with NheI to linearize any plasmid DNA containing an NheI site and treated with phosphatase to prevent circularization. Rep+ and Rep- donors were transformed with this DNA, mated, and plasmid DNA was again isolated from a pool of 25 transconjugants. Samples of plasmid DNA isolated before and after enrichment were analyzed for the presence of the FspI site (Figure 3.2C). If the FspI plasmids were due to insertion of multiple copies of the heteroduplex oligonucleotide, then they would contain an NheI site as well as an FspI site. Digestion

with NheI and treatment with phosphatase increased the proportion of FspI plasmids in the population, so these plasmids are not the result of cloning multiple copies of the heteroduplex oligonucleotide into the test plasmid.

Although DNA due to second-round transfer was detected when Rep- donors are used, this DNA made up only a small proportion of the plasmid DNA in a population (Figure 3.1B). There are two possibilities to explain this low level of observed second-round transfer. The first is that strand replacement in the donor is inefficient, resulting in few second-round transfer strands. The second possibility is that strand replacement in the donor occurs efficiently, but this new strand is not immediately transferred to the recipient cell. In Rep- donors, the number of plasmids available for transfer remains low. The lower number of selectable plasmid copies (one, or at most a few per cell) mean that generally the transferred strand of the plasmid must be replaced before a second round of transfer can be initiated. If the newly synthesized strand were unavailable for immediate transfer, it would result in a lag between rounds of transfer. During such a lag, the first transferred strand would have sufficient time to become established in the recipient before the second round of transfer is completed. Since the NheI and FspI plasmids contain identical replicons, this would prevent FspI plasmids from becoming established in the recipient due to incompatibility with the NheI plasmids.

Is the low proportion of FspI plasmids after matings with Rep- donors due to a lag between rounds of transfer from a single plasmid? Preventing the first round of transfer from becoming established in the recipient should allow subsequently transferred strands to become established even after a lag, since incompatibility would no longer be a possibility. A new heteroduplex oligonucleotide was designed such that the first transferred strand contained a point mutation in *oriV* that prevented vegetative replication of the plasmid (Kim and Meyer 1991). The second transferred strand contained the

unmutated *oriV*. This heteroduplex oligonucleotide was inserted into test plasmid pUT1740 (Figure 2.1), between the ApaI and NgoMIV sites, prior to electroporation into Rep- donor cells. As a result of the *oriV* mutation, the strand transferred first will not establish in the recipient cell (Figure 3.3). Therefore, the second-round transfer strand would become the dominant plasmid in the recipient cell after successful replacement strand synthesis.

In order to estimate the efficiency of transfer for each strand, the NheI/FspI mismatch and oriV-/oriV+ mismatch test plasmids were separately introduced into Rep- donors and mated with Rep+ recipient cells. Transconjugants were selected on TYE supplemented with chloramphenicol and nalidixic acid. In addition, the NheI/FspI mismatch test plasmid was also introduced into Rep+ cells and plated on TYE supplemented with chloramphenicol in order to estimate the number of potential donors in the matings. The frequency of first-round transfer was calculated as the number of transconjugants arising from the NheI/FspI mating, divided by the number of potential donors. The frequency of second round transfer was calculated as the number of transconjugants from the oriV-/oriV+ mating divided by the number of potential donors. The frequency of first and second round transfer was 2.6×10^{-4} and 1.2×10^{-4} transconjugants/donor, respectively. The high frequency of second round transfer relative to first round transfer indicates that strand replacement synthesis occurred efficiently in the Rep- donors since strand replacement in the donor is a prerequisite for second round transfer.

The reason for the eclipse period between rounds of transfer is unclear, but the resulting lag is only observed when very few plasmid molecules are available for transfer. When plasmid replication is not occurring in the donor, the eclipse period results in a pause between subsequent rounds of transfer from the donor, allowing the first round of

transfer to become established in the recipient before a second round of transfer can occur. As second round transfer occurs, it is difficult for the incoming strands to become established in the recipient because of incompatibility with the first round transfer strands. The presence of an eclipse period between rounds of transfer argues against the idea that the plasmid remains associated with the transfer pore while continuously transferring strands to the recipient, as this would result in rapid transfer of multiple strands under conditions in which there is no vegetative replication, and therefore no eclipse period would be seen.

Under conditions in which the plasmid is replicating in the donor, the eclipse period does not delay start of another round of transfer because the transfer pore can select another plasmid to transfer while the previously transferred plasmid is unavailable. The strand first transferred does not have time to become established by extensive replication in the recipient before the next transferred strand arrives, and therefore it cannot exclude the second strand through incompatibility. The ability of the transfer pore to mobilize multiple strands in a short amount of time implies that the plasmids are localized near the transfer pore, possibly due to interaction between MobA relaxase and the TraG coupling protein supplied by R751. Although no such interaction has been directly demonstrated using R1162 proteins, interaction has been observed between the relaxase of pBHR1 and TraG of plasmid RP4 (Szpirer, Faelen, and Couturier 2001). Interaction between MobA molecules bound to *oriT* might also prevent plasmids from diffusing throughout the donor cell (Zhang, Zhang, and Meyer 2003), insuring that multiple copies of the plasmid are available for transfer at any one time.

3.2 THE ORIL PRIMING SITE ENHANCES, BUT IS NOT REQUIRED FOR, REPLACEMENT STRAND SYNTHESIS IN THE DONOR

Initiation of multiple rounds of transfer from a single plasmid molecule indicates that strand replacement occurs in the donor, but does not reveal how this occurs. The R1162 *oriV* contains two single-stranded priming sites, *oriL* and *oriR*, located on opposite strands (Figure 1.1B). No other priming sites utilized by RepB' primase have been found in R1162. Therefore it is reasonable to assume that any RepB'-directed strand replacement synthesis occurring in the donor after a round of transfer would be directed by *oriL*, the priming site located on the strand that remains in the donor after transfer. A test plasmid lacking *oriL* was used to determine if this assumption was correct. In addition, a heteroduplex test plasmid, also lacking *oriL*, was used to better understand the role of *oriL* in strand replacement.

Since plasmids lacking *oriL* would be replicated poorly at best (Becker, Zhou, and Meyer 1996), the plasmid was maintained as a fusion with pBR322. Test plasmid DNA were separated from pBR322 by digestion with BamHI, purified by gel electrophoresis, and ligated to circularize the molecules. This plasmid would also be unstable in a transconjugant cell after transfer. For this reason, the lambda *attP* site was cloned into the test plasmid, and the recipient cells contained a plasmid (pUT1612) expressing lambda integrase. After complementary strand synthesis in the recipient, the incoming test plasmid DNA was rescued by recombination at *attB* in the chromosome.

The first test was to see if *oriL* is required for transfer. pUT1742 (oriL+) and pUT1743 (oriL-) plasmids were introduced into the Rep- donors and mated with Nal^R recipients either expressing or lacking lambda integrase (Int). Table 3.1 shows that the test plasmid is rescued by lambda integrase (lines 1 and 3) and that transconjugants were formed regardless of whether *oriL* was present or not (lines 3 and 4). Transconjugants

can arise after the first round of transfer (Figure 3.2B, line 2), indicating that strand replacement in the donor is not required during conjugation. Formation of transconjugants under conditions (oriL-) where strand replacement in the donor might be hindered is not surprising. However, since it is impossible to distinguish between first- and second- round transfer in this experiment, the possibility that multiple rounds of transfer is occurring in the absence of *oriL* cannot be ruled out.

To distinguish between first- and second- round transfer in the absence of *oriL*, heteroduplex test plasmids that contained an XmnI site on the first strand transferred, and a SapI site on all subsequently transferred strands (Figure 3.4), were used. The heteroduplex was ligated to the oriL+ or oriL- test plasmids, replacing the majority of the pBR322 DNA in the process. The resulting plasmid was then used in the electroporation/mating assay with the lambda integrase recipient where the transferred DNA was integrated into the chromosome. The restriction site in the transferred DNA was determined by amplifying a 1500-bp fragment of the test plasmid, inserted into the recipient chromosome, by whole-cell PCR and digesting this DNA with XmnI or SapI.

Chromosomal DNA from a pooled population of 25 transconjugants was the template for whole-cell PCR. When the test plasmid contained *oriL*, most of the PCR product contained the SapI site (Figure 3.4. lanes 1 and 2), indicating multiple rounds of transfer. On the other hand, when *oriL* was missing, most of the PCR product contained an XmnI site (Figure 3.4, lanes 3 and 4), indicating that DNA from the first-round transfer made up the majority of molecules incorporated into the chromosome. Interestingly, even when *oriL* was missing, some SapI-containing DNA was detected in the PCR product, indicating that multiple rounds of transfer can occur in the absence of *oriL*. The proportion of the transconjugant population arising from second round transfer due to this alternative method of strand replacement is lower than when *oriL* is

present. This indicates that the alternative method is less efficient than the *oriL*-dependent mechanism.

If priming at *oriL* contributes to the frequency of second-round transfers, then it should be possible to complement oriL- by providing the plasmid with another mechanism for initiating complementary strand synthesis. An additional pair of test plasmids, either containing or lacking *oriL*, was constructed to contain a primosome assembly site (PAS) from pBR322. Once the PAS sequence is single-stranded, it forms a hairpin structure recognized by PriA, which in turn directs the formation of a primosome capable of initiating strand synthesis (Marians 1999). A heteroduplex oligonucleotide that formed either an FspI site (first-round transfer) or an NheI site (second-round transfer) after transfer was ligated into each test plasmid. When the oriL+ heteroduplex test plasmid was used in an electroporation/mating and amplified from a pool of 25 transconjugants, the majority of the PCR product contained an NheI site (Figure 3.4, lanes 5 and 6), indicating that second-round transfer was occurring efficiently. In the absence of *oriL*, second-round transfer remained efficient (Figure 3.4, lanes 7 and 8) confirming that initiation of complementary strand synthesis, in this case by a PAS sequence, can increase the frequency of second-round transfer. I interpret these data to mean that synthesis of the missing strand in the donor can be initiated from the PAS sequence, replacing *oriL* in strand replacement synthesis.

The small amount of second-round transfer seen in the absence of *oriL* (Figure 3.4, lane 4) is possibly initiated by extension of the free 3' end resulting after MobA nicks the transfer strand or by the action of a host-encoded mechanism similar to the one responsible for allowing vegetative replication of R1162 molecules lacking one priming site (Zhou and Meyer 1990), but this mechanism of strand replacement is less efficient than priming at *oriL*.

3.3 THE PRIMING SITES OF *ORIV* ARE NOT REQUIRED FOR PLASMID ESTABLISHMENT IN THE RECIPIENT AFTER TRANSFER INTO *E. COLI*

Although *oriL* is not required during plasmid transfer, it is possible that the oppositely oriented priming site, *oriR*, is required. Since *oriR* is oriented for priming the complement to the transferred strand, it is the best candidate for strand replacement synthesis in the recipient. The observation that the linkage of primase to MobA is required for detectable transfer under non-replicative conditions (Henderson and Meyer 1999) supports this hypothesis. The role of priming sites in establishment of the transferred strand was studied in two ways. The first approach used test plasmids lacking either *oriR* or *oriR* and *oriL*. The second approach used donor cells containing a relaxase that lacks a linked primase region, and is therefore unable to utilize the priming sites on the test plasmid.

A new test plasmid was created that lacked *oriR*, but retained all other components of *oriV* (pUT1750). As expected, this plasmid was unstable, so the test plasmid was again maintained as a fusion to pBR322 until utilized in an electroporation/mating assay. After transfer of the test plasmid, any plasmid capable of replacement strand synthesis was rescued by integration into the chromosome by lambda integrase.

Introducing the oriR- test plasmid into Rep- donor cells and mating with recipient cells expressing lambda integrase produced unexpected results. Deletion of *oriR* resulted in only a 1.8-fold reduction in the number of transconjugants compared to when *oriR* was present (Table 3.2, lines 3 and 4). This decrease was less than expected and indicates that *oriR* is not required for initiation of replacement strand synthesis. Very few transconjugants were recovered when lambda integrase was absent, indicating that the transconjugants formed in the absence of *oriR* were due to integration of the test

plasmid into the recipient chromosome and not from spontaneous resistance to chloramphenicol by the recipient cell (Table 3.2, lines 1 and 2). The removal of both *oriL* and *oriR* resulted in a 2.1-fold decrease in the number of transconjugants (Table 3.3, lines 1 and 2); similar to the decrease when *oriR* alone is removed (Table 3.2, lines 3 and 4).

One possible explanation for the small reduction in the number of transconjugants after removal of the priming sites is that the R751 primase, TraC, is initiating replacement strand synthesis on R1162 DNA in the recipient. To test the role of TraC in transconjugant formation, the *traC* gene of R751 was inactivated by insertion of a non-polar, promoterless kanamycin resistance gene (Menard, Sansonetti, and Parsot 1993). TraC primase is not required for conjugative transfer of RP4 into *E. coli* (Lanka and Barth 1981), so the loss of *traC* did not prevent the use of R751 as a helper plasmid in the electroporation/mating assay. In the presence of this altered plasmid, the oriR- test plasmid was still capable of producing transconjugants (Table 3.2, lines 5 and 6). In fact, removing TraC resulted in a 4-fold increase in the number of transconjugants. The reason for this increase in the absence of TraC is unknown.

The formation of transconjugants in the absence of priming sites does not rule out the possibility that the MobA-linked primase is contributing to initiation of strand replacement. Perhaps the primase is able to recognize a site other than *oriR* in the recipient. If this is the case, then inactivation of the primase region of MobA should prevent the formation of transconjugants. MobA containing a primase-inactivating, in-frame insertion (in the plasmid pUT1790) was used to mobilize oriL+/oriR+ or oriL-/oriR- test plasmid in an electroporation/mating assay. Inactivation of the primase reduced the number of transconjugants whether or not the priming sites were present (Table 3.3).

If RepB' were involved in priming, even in the absence of *oriL* and *oriR*, it should be possible to bypass the requirement for RepB' by providing an independent means of initiation. A new test plasmid was created that contained a PAS sequence in place of *oriR*. This new plasmid is stable in the presence of RepA and RepC, indicating that the PAS sequence is able to substitute for the missing priming site as long as there is a mechanism in place to expose the PAS sequence as single-stranded DNA (Ray et al. 1982; Scherzinger et al. 1991). After transfer of the plasmid to the recipient cell, the single-stranded PAS sequence should be able to initiate replacement strand synthesis. When the oriL+/PAS test plasmid was used in an electroporation/mating assay, transconjugants were formed at amounts similar to when *oriR* was present (Table 3.3, lines 1 and 3). However, when the same plasmids were transferred from donors expressing the inactivated primase, fewer transconjugants were formed than when active primase was present (Table 3.3, lines 4 and 6). This suggested that inactivating the primase reduced the number of transconjugants because of poor transfer instead of poor initiation of strand replacement.

Since the mutation in the primase region of MobA affected transfer of the test plasmids, we focused on the transfer system of pSC101. Although the mechanisms of vegetative replication of pSC101 and R1162 are unrelated, the relaxases of these two plasmids are very similar. However, the pSC101 MobA is not linked to a primase at its C-terminus. In this sense, it is an example of a primase-deficient member of the R1162 MobA family. The *oriTs* of R1162 and pSC101 are also very similar, both containing a large inverted repeat and nearly identical cores. In fact, the R1162 MobA can utilize the pSC101 *oriT* to mobilize plasmids at a high frequency (Meyer 2000). However, the pSC101 MobA is inactive on the R1162 *oriT*. In order to utilize the pSC101 MobA system in the electroporation/mating assay, a new test plasmid was designed that was

identical to the oriL+/oriR+ test plasmid, pUT1735, except the R1162 *oriT* was replaced by a pSC101 *oriT*. Since test plasmids containing the pSC101 oriT have not been used in electroporation/mating assays in the past, these new plasmids were first tested for their mobility from Rep- donors expressing either R1162 MobA or pSC101 MobA (Table 3.4). As expected, R1162 MobA was able to mobilize test plasmids containing either *oriT* (Table 3.4, lines 1 and 2). The pSC101 MobA was only able to mobilize the test plasmid containing the pSC101 *oriT* (Table 3.4, lines 3 and 4).

pSC101 *oriT* test plasmids, with or without *oriL* and *oriR*, were introduced into donors expressing pSC101 MobA. Transfer of either plasmid resulted in transconjugants, indicating that the priming sites were not needed for transfer (Table 3.5, lines 1 and 2). Since the pSC101 MobA does not contain a primase region, these results also indicate that MobA-linked primase is not required. Unlike the previous experiment using R1162 *oriT* plasmids, (Table 3.3, lines 1 and 2), removal of both priming sites from pSC101 *oriT* plasmid did not change the number of transconjugants (Table 3.5, lines 1 and 2). Thus, without a MobA-linked primase, *oriL* and *oriR* cannot contribute to the frequency of transfer. However, the appearance of transconjugants in the absence of both primase and priming sites (Table 3.5, line 2) again indicates that there is another mechanism responsible for initiation of strand replacement in the recipient. The experiment was repeated using donor cells that harbor the TraC- R751 helper plasmid. In agreement with the previous results with R1162 (Table 3.3, lines 5 and 6), inactivation of TraC did not prevent the formation of transconjugants (Table 3.5, lines 3 and 4). In fact, the number of transconjugants again increased in the absence of TraC (Table 3.5, lines 2 and 4). Expressing unlinked primase in the recipient did not increase the number of transconjugants (Table 3.5, lines 5 and 6).

I conclude that priming sites *oriL* and *oriR* are not required for initiation of strand replacement after transfer into the recipient. However, it should be noted that the number of transconjugants was always higher when the test plasmid contained the priming site and MobA-linked primase (Table 3.2, line 3; Table 3.3, line 1; Table 3.4, lines 1 and 2) in relation to test plasmids lacking priming sites or donors lacking MobA-linked primase. This is evidence that *oriR* can participate in initiation of strand replacement, but, like *oriL*, is not required. The formation of transconjugants in the absence of priming sites and MobA-linked primase (Table 3.5 line 2) indicates that in *E. coli* there is a host-encoded mechanism for initiating strand replacement in the recipient.

3.4 TRANSFER OF R1162 INTO *SALMONELLA* DOES NOT REQUIRE TRAC PRIMASE

Lanka and Barth (1981) noted that the TraC primase of RP4, a plasmid related to R751, was required during conjugative transfer of either RP4 or IncQ plasmid R300B into *Salmonella typhimurium* but not into *E. coli*. They concluded that this requirement might be the result of inefficient, host-directed initiation of replacement strand synthesis in *Salmonella*. If this is the case, then is there as well poor host-directed replacement strand synthesis of R1162 DNA?

One possible drawback to using *Salmonella* as a recipient in mating is that the *Salmonella* chromosome might not be a target for lambda integrase-directed integration of the test plasmids. The LamB outer membrane protein encoded by *Salmonella* is not a receptor for bacteriophage lambda (Schwartz and Le Minor 1975). Since *Salmonella* is not a natural host for lambda, it may not posses a functional *attB* attachment site for site-directed integration into the chromosome. According to the published genome of *Salmonella typhimurium* LT2 (McClelland et al. 2001), the *Salmonella* chromosome contains a site located near the *gal* operon homologous to *E. coli attB* in all but one base (Figure 3.5, top). To see if *attP* plasmids could integrate at this site, the test plasmid,

pUT1735, was transferred from an *E. coli* donor to either *E. coli* or *Salmonella* recipients. Both types of recipients carried the lambda integrase expression plasmid. The transfer frequency from *E. coli* to *E. coli* was 2.7 x 10^{-2} transconjugants/donor. The *E. coli* to *Salmonella* mating resulted in 1.7 x 10^{-3} transconjugants/donor. The lower frequency into *Salmonella* could be due to host restriction. Integration of the test plasmid into the *Salmonella* chromosome was confirmed by whole-cell PCR (Figure 3.5, bottom). Unmated *Salmonella* recipient DNA was used as a control to determine if the primer pair produced any product from the *Salmonella* chromosome. PCR product of the expected size was obtained only from transconjugant chromosomes, suggesting that the *Salmonella* chromosome does contain a viable *attB* site in which a test plasmid can be integrated.

RP4 TraC is required for efficient transfer of R300B into *Salmonella* (Lanka and Barth 1981). Perhaps this requirement reflects the lack of an efficient host priming system. R751 and RP4 Tra systems are related; the R751 TraC primase could be required for transfer of the test plasmid into *Salmonella*. To test this possibilty, sets of intraspecific (*E. coli* x *E. coli* or *Salmonella* x *Salmonella*) and interspecific (*E. coli* x *Salmonella*) matings were performed (Figure 3.6). The donors contained R751 or the TraC-deficient derivative as well as the R1162 *oriT* test plasmid, pUT1735, and a helper plasmid expressing the R1162 Mob and Rep proteins. In the case of the *E. coli* x *Salmonella* mating, a restrictionless *Salmonella* strain (LB5000 Nal^r) was used. The transfer frequencies for pUT1735 and R751 were calculated by dividing the number of transconjugants by the number of donors for each mating.

Removing TraC primase slightly increased (1.6-fold) the transfer frequency for R751 (*E. coli* x *E. coli* matings, Figure 3.6A). In contrast, inactivating TraC decreased the transfer frequency when *Salmonella* was the recipient (Figure 3.6B, C). The transfer frequency was reduced 3.1-fold for *Salmonella* donors and 4.7-fold for *E. coli* donors

(Figure 3.6B,C). The impaired transfer of R751 into *Salmonella* in the absence of TraC agrees with the results previously reported by Lanka and Barth for RP4 in which loss of TraC activity resulted in a 25-fold decrease in transfer frequency. The larger effect reported by Lanka and Barth might be due to their use of random transposon insertions to inactivate TraC, resulting in a polar effect on downstream *tra* genes.

Test plasmid pUT1735 was used to determine if TraC is required for efficient transfer of R1162-based plasmids into *Salmonella*. The transfer frequency of pUT1735 into the *E. coli* recipient increased 4-fold (Figure 3.6D) when TraC was removed, agreeing with previous data (Table 3.5, lines 1 and 3). Inactivation of TraC also increased the transfer frequency of pUT1735 into *Salmonella* (Figure 3.6E, F). Transfer from *E. coli* to *Salmonella* increased 2.5-fold while transfer from *Salmonella* to *Salmonella* increased by 3.6-fold. This disagrees with the earlier observations of Lanka and Barth (1981) in which the transfer of IncQ plasmid R300B was negatively affected by the loss of RP4 TraC. The difference between our results and those of Lanka and Barth might be due to our use of R751 instead of RP4. Another possibility is that the transposon insertions used by Lanka and Barth to create TraC mutants might have had a polar effect on expression of other Mob genes, resulting in a lower transfer frequency of R300B. In any case, the increased transfer of the test plasmid from donors lacking TraC again shows that, unlike R751, the R1162-based test plasmid does not require TraC for transfer into *Salmonella*.

3.5 R1162 STRAND REPLACEMENT IN *SALMONELLA* RECIPIENTS REQUIRE A PLASMID-ENCODED PRIMING SYSTEM

The requirement of a conjugative primase (TraC) for efficient transfer of R751 into *Salmonella* (Figure 3.6, line 1) might indicate that the host-encoded strand replacement mechanism in *Salmonella* is less efficient than in *E. coli.* This should allow

us to study the role of R1162-directed strand replacement in the recipient without the high background seen when *E. coli* was used. Electroporation/mating assays using *Salmonella* donors and recipients were used to determine the contribution of R1162 primase and priming sites in plasmid establishment after transfer.

Intraspecific matings were performed between *E. coli* or *Salmonella* donors and recipients to determine if strand replacement is *oriV*-independent in *Salmonella* as well as in *E. coli*. Donors containing R751 TraC- were used since these gave the greater transfer efficiency (Figure 3.6, line 2). Rep- donor cells were electroporated with either R1162 oriT/oriV+, R1162 oriT/oriV-, pSC101 oriT/oriV+ or pSC101 oriT/oriV- test plasmids. The donor cells expressed either R1162 MobA or pSC101 MobA while the recipient cells expressed lambda integrase to rescue transferred DNA completing strand replacement (Figure 3.7).

When the mating pair was *E. coli*, deletion of *oriV* had little effect on the formation of transconjugants, in agreement with Table 3.3 (Figure 3.7A, C, E). Likewise, using pSC101 MobA to mobilize test plasmids between *E. coli* showed that a primase-deficient system is still capable of giving rise to transconjugants (Figure 3.7, line 3). However, when the ori*V*- test plasmids were used in the *Salmonella* system, the number of transconjugants was reduced compared to the oriV+ test plasmid (Figure 3.7B, D, F). When R1162 MobA was present in the donor, the number of transconjugants decreased about 12-fold in the absence of *oriV* (Figure 3.8B, D), indicating that the priming sites are important during transfer into *Salmonella*. When *Salmonella* donors contained pSC101 MobA, the number of transconjugants was reduced by 2.3-fold when *oriV* was removed (Figure 3.7F). However, the total number of transconjugants was low, regardless of whether *oriV* was present or absent (Figure 3.7, lines 2 and 3). Taken as a whole, the results from the *Salmonella* electroporation/mating assay is evidence that

the primase is needed for efficient formation of transconjugants. However, the primase was not absolutely required since transconjugants were still formed in its absence (Figure 3.8, line 3). This is in agreement with the idea that *Salmonella* does have a host-encoded mechanism for strand replacement, but it is less efficient than the mechanism in *E. coli*.

If R1162 primase is initiating strand replacement in the recipient, the logical site of action is *oriR*. After transfer, *oriR* would be single-stranded and positioned in the correct orientation for initiation of replacement strand synthesis in the recipient. This priming site is not required for formation of transconjugants in *E. coli* (Table 3.2), but the less efficient host encoded mechanism in *Salmonella* might make *oriR* more important for plasmid establishment. To test this, R1162 *oriT* test plasmids lacking either *oriL* or *oriR* were used. For *E. coli* matings, deletion of either priming site had little effect on the average of transfer (Figure 3.8A, C). However, deleting *oriR*, decreased the number of transconjugants formed in matings with *Salmonella* (Figure 3.8D). Removal of *oriL*, on the other hand, had little effect on the number of transconjugants (Figure 3.8B). This indicates that in *Salmonella* the transferred plasmid must rely more heavily on *oriR* for establishment.

Donors containing pSC101 MobA produce few transconjugants in *Salmonella* (Figure 3.7, line 3), presumably because the pSC101 MobA does not contain a primase able to utilize *oriR* to initiate strand replacement in the recipient. It might be possible to complement this lack of primase activity by expressing R1162 primase in the recipient. Test plasmid DNA containing a pSC101 *oriT* and an R1162 *oriV* was introduced into donors harboring the pSC101 MobA protein and mated with recipients expressing lambda integrase either alone or in the presence of R1162 primase. The expressed primase was RepB', not the full-length relaxase. When *E. coli* cells were used, the

addition of primase had little effect (Table 3.6, lines 1 and 2). This was expected since earlier experiments indicated that, in *E. coli*, transconjugants are formed in the absence of the plasmid encoded priming system (Figure 3.7, line 3). However, when *Salmonella* cells were used, the addition of primase resulted in a 3-fold increase in the number of transconjugants (Table 3.6, lines 3 and 4). This small increase was less than expected; the number of *Salmonella* transconjugants recovered when primase was supplied in the recipient (Table 3.6, line 4) was approximately 7-fold lower than when MobA-linked primase was used (Figure 3.7, line 1). The low level of complementation might be a result of the primase being in the unlinked form. When the primase is linked to the relaxase, it might be in position to initiate strand replacement synthesis quickly after transfer due to the proximity of *oriT* and *oriV*.

Despite the low level of complementation by RepB' in the *Salmonella* recipients, *oriR* and the primase region of MobA obviously play a role in plasmid establishment under conditions in which the host encoded mechanism is inadequate. The fact that the MobA-linked primase and *oriR* are used for plasmid establishment in *Salmonella* (Figure 3.7 lines 2 and 3; Figure 3.8, lines 1 and 3) is evidence that the plasmid-encoded primase can initiate replacement strand synthesis at *oriR*. However, the host-encoded mechanism used by the plasmid to bypass efficiently this requirement in *E. coli* remains unclear.

3.6 *E. COLI* DNAG MIGHT PLAY A ROLE IN HOST-ENCODED STRAND REPLACEMENT

Although the above experiments demonstrate that an efficient host-encoded mechanism for strand replacement synthesis exists in *E. coli*, it does not shed any light on the nature of that mechanism. Because the transferred DNA is single-stranded, any priming sites might be in their active form upon arrival in the recipient cell. A replication-deficient M13 phage was used to search for fragments of the oriV- test

plasmid that might act as initiation sites for strand replacement. The possibility that *E. coli* DnaG was involved in strand replacement was also explored.

In an effort to identify cryptic initiation sites, fragments of the oriV- test plasmid were created by digestion of the test plasmid with Tsp509I and introduced into the EcoRI site of phage M13Δe101. This phage is missing the initiation site normally used by M13 for strand replacement (Ray et al. 1982), and as a result, forms small plaques. It has been shown previously that insertion of an initiation site into the EcoRI site of this phage results in the formation of larger plaques (Yakobson et al. 1990). However, no large plaques were recovered after insertion of test plasmid DNA into the EcoRI site. As a control, the experiment was also performed with a plasmid identical to the oriV- test plasmid except containing two oppositely oriented PAS sequences were the priming sites are normally located. Insertion of Tsp509I fragments from this PAS plasmid into the EcoRI site of M13Δe101 resulted in larger plaques and DNA isolated from these larger plaques was found to contain a PAS.

One possible explanation for the lack of complementation by the oriV- test plasmid fragments is that the site used by the host-encoded mechanism is only weakly active. If this were the case, complementation of M13Δe101 by the test plasmid fragments would be inefficient. To increase the chance of finding an initiation site, an enrichment was performed in which phage particles from plaques, formed after transfection of JM103 with M13Δe101 harboring oriV- test plasmid fragments, were washed off the plate and used to transfect fresh JM103 cells. This enrichment was carried out for three cycles, but no large plaques were seen after the final transfection. Again, the PAS test plasmid was used as a control and resulted in numerous large plaques (Figure 3.9E). The DNA from these phage particles all contained a PAS site at the EcoRI site as expected.

Interestingly, the M13Δe101 phage, with no supplementary DNA inserted into the EcoRI site, developed small plaques after transfection of JM103, but large plaques when JM103 also harbored R751 (Figure 3.9A, B). The large plaques required a functional TraC primase (Figure 3.9C). When RK2, an IncP plasmid related to R751, was resident in the transfected cell, the plaques were of an intermediate size (Figure 3.9D). While these data do not shed any light on the mechanism of host-encoded strand replacement, it does indicate that TraC primase, which displays a degree of specificity that prevents it from suppressing a *dnaG*(ts) mutation (Lanka and Barth 1981), is capable of priming a substrate other than R751. However, TraC does not account for R1162 strand replacement since inactivation of TraC failed to prevent initiation of strand replacement (Figure 3.6D).

One candidate for initiating host-directed strand replacement is DnaG primase. Although *E. coli* and *Salmonella* DnaG proteins share 86.2% identity, it is possible that the *Salmonella* DnaG is somehow less active in initiation of strand replacement than its *E. coli* counterpart, resulting in fewer transconjugants in the absence of a plasmid-encoded strand replacement mechanism. When recipient cells contained the temperature sensitive *dnaG308(ts)* mutation (Marinus and Adelberg 1970), the number of transconjugants was unaffected when the recipient cells were shifted to the non-permissive temperature. However, it was unclear if the DnaG was completely inactivated by the temperature shift, or if a portion of the DnaG within the recipient cells remained active. If the latter is the case, then the appearance of transconjugants may be a result of this residual activity.

A different approach was used to observe the role of *E. coli* DnaG primase more directly. A low-copy number plasmid expressing *dnaG* from an IPTG-inducible promoter was constructed. This plasmid complemented the temperature-sensitive *dnaG*

mutation in the absence of IPTG, suggesting leaky expression. This DnaG-expressing vector was introduced into *E. coli* and *Salmonella* cells harboring the lambda integrase vector. These strains were then used in a series of electroporation/mating assays.

Test plasmids containing pSC101 *oriT* were electroporated into pSC101 MobA donors that were then mated with recipients containing either the DnaG expression plasmid or a vector-only control (pWSK129). When *E. coli* cells were used, the presence of additional DnaG in the recipient had little effect on the number of transconjugants compared to the negative control (Table 3.7, lines 1 and 2). Either the amount of DnaG in *E. coli* is not rate-limiting, in which case additional DnaG will not increase the efficiency of replacement strand synthesis, or DnaG primase does not prime strand replacement synthesis. When the same mating was performed with *Salmonella* cells there was a 3.9-fold increase in the number of transconjugants when *E. coli* DnaG is expressed in the recipient cell (Table 3.7, lines 3 and 4). This increase in the number of transconjugants is lower than the increase due to a primase-linked MobA in *Salmonella* (10-fold compared to MobA lacking a linked primase (Figure 3.7, lines 2 and 3)), but is similar to the increase when unlinked primase was supplied in the recipient (Table 3.6, line 3 and 4). One possible explanation for these results is that in both cases (RepB' or DnaG) the primase is unable to find the single-stranded DNA in the recipient after a round of transfer. Without the benefit of being linked to the transferred molecule, the primase is at a disadvantage for finding substrates as they enter the cell. When compared to the results from when unlinked R1162 primase was supplied in the recipient, the increase in the number of transconjugants when *E. coli* DnaG is supplied in *Salmonella* recipients (Table 3.7, lines 3 and 4) agrees with the possibility that DnaG is able to initiate strand replacement in the recipient.

Using *Salmonella* in the electroporation/mating assays has been helpful in determining that the R1162 priming system can initiate strand replacement after transfer into the recipient, but the mechanism used in *E. coli* to bypass the R1162 system remains unclear. The results from the M13Δe101 assay clearly indicate that R1162 does not contain any sites, other than *oriL* and *oriR*, capable of initiating strand replacement in an efficient manner. *E. coli* DnaG is a candidate for initiating replacement strand synthesis in the recipient since supplying this protein in *Salmonella* recipients increases the number of transconjugants by the same factor as when RepB' is supplied, but this does not exclude the possibility that additional, or more efficient, host encoded mechanisms are involved.

Chapter 4. Results: Type IV transport of MobA

4.1 MobB is required for efficient transfer of MobA to the recipient cell

Aside from initiating strand replacement synthesis in the donor and recipient, another possible role of MobA-linked primase might be as a transfer signal for the full-length protein during transfer of the nucleoprotein complex through the R751 pore. Several labs studying type IV transfer systems have suggested that the C-terminal region of an exported substrate is critically important for export, and must contain positively charged amino acids, usually arginine, 10-15 residues from the C-terminal end of the protein (Vergunst et al. 2005; Luo and Isberg 2004). This conclusion was based on the fact that when the C-terminal portion of known substrates of the Vir system of *Agrobacterium* and the Dot/Icm system of *Legionella* are fused to Cre recombinase, the chimeric proteins are transferred to the recipient cell. In fact, the C-terminal 48 amino acids of RSF1010 MobA were found to be sufficient to translocate Cre through the *Agrobacterium* VirB/D4 system (Vergunst et al. 2005).

Transport of relaxases through pores encoded by transmissible plasmids has been shown (Draper et al. 2005; Luo and Isberg 2004), but the locations of the transport signals have not been clearly defined. There is evidence that the signal responsible for relaxase transport may not reside solely in the C-terminus. For example, the C-terminal half of R1162 MobA can be removed without drastically affecting transfer (Brasch and Meyer 1986).

I developed a system to monitor the transport of MobA through the R751 pore. The system was based on previous work done with Cre recombinase fusions (Luo and Isberg 2004). A Cre sensor was designed that would allow detection of Cre-MobA

fusions in recipient cells (Figure 4.1). This sensor consists of a nonpolar, promoterless kananycin resistance gene (*aph*), flanked with *lox* sites oriented in the same direction, located within the 5' region of *lacZα*. *E. coli* cells containing a single, chromosomal copy of this Cre sensor were used as recipients in matings with cells expressing Cre-MobA fusions and harboring the R751 helper plasmid. Prior to mating, the recipient cells were kanamycin-resistant and formed white colonies in the presence of X-gal. If Cre-mediated recombination occurred at the sensor, however, the cell formed blue colonies in the presence of X-gal, due to excision of the kanamycin resistance gene between the two *lox* sites.

All Cre fusions used in this study were tested for their ability to activate the Cre sensor prior to their use in the following transport assay. Donor cells containing various Cre-MobA fusions and the R751 helper plasmid, which encodes resistance to trimethoprim, were mated with streptomycin-resistant, Cre-sensing recipient cells. Trimethoprim- and streptomycin-resistant transconjugants, created by successful transfer of R751, were plated on selective medium containing X-gal. The proportion of the population receiving Cre-fused transfer products was calculated from the number of transconjugants that were blue. Transport of unfused Cre was not observed in this assay (data not shown).

Cre was first fused to the N-terminal end of full-length MobA and tested with the Cre transfer assay described above. 0.097% of the transconjugants were blue on X-gal (Figure 4.2, line 1). This indicates that a relatively low proportion of R751-containing transconjugants received enough Cre-fused protein to trigger the Cre sensor in the recipient. It should be noted, however, that Cre-recombination within the recipient requires a minimum of four Cre-MobA molecules (Hoess and Abremski 1984). Thus, the number of cells receiving MobA is probably being underestimated in this assay.

The open reading frame for MobB is located within the open reading frame for MobA (Figure 1.1). Therefore, the plasmid expressing the Cre-MobA fusion is also expressing MobB. To determine the affect of MobB on the transport of the Cre-MobA fusion, a new fusion was created containing an in-frame deletion within the region encoding MobB (Figure 4.2, line 2). This deletion causes a 1000-fold decrease in the transfer efficiency of R1162 (expressed as transconjugants/donor) (Perwez and Meyer 1996). When this mutation was present, blue transconjugants were undetectable (<0.0011%). When MobB was supplied in *trans*, blue transconjugants were again formed, and at a percentage (0.11%) similar to that of the full-length fusion (0.097%). Thus, MobB is clearly important for transport of MobA by the type IV transfer pore of R751. Since this assay is performed in the absence of *oriT*, the mechanism must be at least partially distinct from the previously reported stabilization of the relaxosome by MobB (Perwez and Meyer 1999). A fusion between MobB and Cre (Figure 4.2, line 6) did not result in blue transconjugants (<0.0014%), so MobB is not acting as a carrier for MobA.

Two additional fusion proteins containing in-frame, internal deletions were constructed. One contained a deletion entirely within *mobB* and removed amino acids 281 through 323 in MobA (Figure 4.2, line 3). The other fusion contained a deletion extending from *mobB* toward the N-terminal region of MobA containing the relaxase and removed amino acids 224 through 323 (Figure 4.2, line 4). The first deletion, in R1162, can be complemented for transfer by providing MobB in *trans*, while the second cannot, presumably due to impaired nicking of DNA (Perwez and Meyer 1999). When these mutations were tested in the Cre transfer assay, neither fusion formed blue transconjugants in the absence of MobB. However, when MobB was supplied in *trans*, the Cre fusion containing the first deletion resulted in 0.09% recombination, similar to

full-length MobA, while the Cre fusion containing the second deletion resulted in 0.63% recombination, a 6-fold increase over full-length MobA. Transport of this fusion indicates that nicking and transport can be functionally unlinked. The higher recombination frequency might indicate that the protein folds so that the fusion is more efficiently transported or the Cre activity is enhanced. Regardless, this is further evidence of a role for MobB in transport of MobA since the elevated percentage of recombination is observed only in the presence of MobB.

One drawback to using the MobB deletions described above is that the structure of MobA is also affected. To further illustrate the requirement of MobB for efficient transfer of Cre-MobA under conditions in which the structure of MobA is not altered, a MobA mutant that no longer co-expressed MobB was created (Figure 4.2, line 5). Six point mutations, silent in *mobA*, remove the start codon for *mobB*, as well as alter the purine content in the sequence to the 5'-side of the *mobB* translation start site. This mutation did not produce blue transconjugants in the assay (<0.0012%). However, when MobB was supplied in *trans*, the percentage of blue transconjugants was restored to a value (0.13%) similar to wild-type MobA. This is strong evidence that MobB is required for efficient transport of MobA through the R751 pore, but does not offer any insight to how this is accomplished. Identifying the signal(s) responsible for recognition of MobA by the R751 pore might help clarify the role of MobB, aside from stabilization of the relaxosome, during conjugation.

4.2 THE TWO FUNCTIONAL DOMAIN OF MOBA CAN BE TRANSPORTED INDIPENDENTLY

Recent studies of type IV protein transport have suggested that the transport signal is located at the C-terminus of the protein targeted for transport (Luo and Isberg 2004; Vergunst et al. 2005; Nagai et al. 2005). More recent studies suggest that there

can be internal signals as well, in addition to signals found at the C-terminus (Schulein et al. 2005). Although the primase domain of MobA is not required for plasmid transfer (Brasch and Meyer 1986), it might still contain a type IV transport signal. In fact, Vergunst, et. al., showed that a fusion between Cre and the C-terminal 48 amino acids of MobA can be transported through the Vir pore (Vergunst et al. 2005) into plant cells. For this reason, a fusion identical to used the one used by this group was constructed to determine if this fragment of MobA contains a transport signal recognized by the R751 pore (Figure 4.3, line 3). The fusion was not transported at detectable levels through the R751 transfer pore, even in the presence of MobB. Thus, the signals recognized by the R751 and Vir type IV transport systems are not identical.

A signal active with the R751 transfer pore might be located elsewhere in the primase region of MobA. The entire primase domain of MobA, encoded by the *repB'* gene, was fused to the C-terminal end of Cre (Figure 4.3, line 4). This fusion protein was not transported, even in the presence of MobB. One possible explanation for this is that the fusion of Cre to RepB' might have destabilized the primase or caused it to misfold. To determine if the primase portion of this fusion is functional, a primase-deficient derivative of R1162 was introduced into the donor strain containing Cre-RepB'. The primase-deficient plasmid was stabilized in the presence of the fusion, indicating that the structure of RepB' is sufficiently intact to be a functional primase.

A fusion was created that consisted of Cre fused to the primase region but separated by an additional 64 amino acids of MobA (Figure 4.3, line 5). The 64 amino acids are encoded by the DNA immediately upstream of the *repB'* start site, and overlapping the 3' end of the *mobB* gene. Remarkably, when this new fusion was tested for transport it resulted in recombination in 29.92% of the transconjugants; a frequency more than 300 times greater than with the fusion containing full-length MobA (Figure

4.3, lines 1 and 5). Again, transport of this fusion required MobB. The high frequency of transport is not due to increased stability of the fusion protein. Western blot analysis of cleared lysates derived from donor cells containing pUT1881 (expressing Cre fused to full-length MobA) and pUT1903 (expressing Cre fused to the C-terminal half of MobA) revealed that the fusions encoded by these plasmids are expressed at similar levels (Figure 4.4, lanes 4 and 5). Furthermore, this fusion was able to stabilize a primase-deficient form of R1162, indicating that the primase domain of this protein is folded correctly, thus ruling out the possibility that the increase in transport was caused by misfolding of the protein and consequent exposure of an alternate transport signal. It is possible that Cre activity can be altered when fused to MobA, resulting in lowered recombination rates. However, the high transport frequency of Cre fused to the C-terminal half of MobA indicates that Cre can retain a high level of activity while part of a fusion.

The high frequency of recombination could be the result of either increased spacing between Cre and RepB', or the presence of a signal located in the linker region. To determine if increased recombination was the result of spacing, a 64-amino acid fragment of the kanamycin resistance peptide encoded by pACYC177 (Aph, amino acids 66 through 129) was placed between Cre and full length MobA (Figure 4.3, line 2) or Cre and RepB' (Figure 4.3, line 6). Even though the addition of the extra amino acids between Cre and full-length MobA increased the percentage of blue transconjugants by more than 20-fold (Figure 4.3, lines 1 and 2), the same amino acids did not increase the frequency of transport when placed between Cre and RepB' (Figure 4.3, lines 4 and 6). Different 64 amino acid linkers, all derived from the same kanamycin resistance peptide, were tested, but none were able to replace the native linker region (data not shown).

The specific requirement for the native linker region during transport of the primase fragment implies that this region contains at least part of a transport signal. To see if this linker region alone was sufficient, it was fused to Cre (Figure 4.3, line 9). This fusion did not result in any blue transconjugants (<0.0017%). Due to its small size, the linker peptide may have been subjected to degradation before it could be transported. To rule out this possibilty, the kanamycin resistance peptide from pACYC177 was fused to the C-terminal end of the Cre-linker fusion (Figure 4.3, line 7). However, this fusion was also unable to produce blue transconjugants (<0.0018%). The portion of this fusion encoding kanamycin resistance was functional as the donor cells expressing this fusion were kanamycin-resistant. However, the possibility that the linker was altered or sequestered by the two other domains of this fusion (Cre and Aph) was not ruled out by this experiment. Taken as a whole, the above data suggest that the 64 amino acids preceding the primase domain, which will be referred to as the P-site (primase site), contains at least part of a signal that is required for transport of the primase fragment of MobA.

Since the P-site could not by itself promote transport, there was still a possibility that the C-terminal end of RepB' was required. The C-terminal 110 amino acids of the highly transportable fusion protein consisting of the P-site and RepB' were removed by inserting an oligonucleotide containing stop codons in all three reading frames into a unique BlpI site (Figure 4.3, line 8). Transport of this truncated fusion was found to be lower than the parent fusion (Figure 4.3, lines 5 and 8) but still higher than the fusion containing full-length MobA (Figure 4.3, lines 1 and 8). This indicates that the C-terminal part of MobA (encoded by *repB'*) is not required for transport through the R751 pore.

Transport of MobA containing a truncation of the C-terminus agrees with previous data in which the first 284 amino acids of MobA were sufficient for transfer of a plasmid containing the R1162 *oriT* (Brasch and Meyer 1986). This study also reported that the first 204 amino acids of MobA were unable to transfer a similar plasmid. Subsequent studies indicated that the smaller MobA fragment retained nicking and ligating activity (Becker and Meyer 2002), indicating that the lack of plasmid transfer was not due to an inability of this MobA to correctly process DNA. These data might give us insight into the location of a possible transport signal located in the relaxase domain of MobA. When the larger MobA fragment was fused to Cre (Figure 4.3, line 11), blue transconjugants were observed (0.47%), but only when MobB was present in the donor cells. However, fusion of the smaller fragment of MobA to Cre (Figure 4.3, line 12) resulted in no blue transconjugants (<0.0011). These results indicate that, as is the case with the primase fragment, there is at least part of a signal located between residues 205 and 284 of the relaxase fragment, which will now be referred to as the R-site (relaxase site). Like the P-site, the R-site is required for transport, but is unable to promote transport when fused to Cre (figure 4.3, line 10), indicating that it too might be part of a larger signal. The P-site and the R-site both lie outside the catalytic regions of the peptide and at least partially overlap the MobB-encoding region.

Interestingly, although the relaxase domain of MobA (Figure 4.3, line 11) and the primase domain of MobA (Figure 4.3, line 5) do not share any overlapping sequence, both require MobB for transfer. This indicates that MobB is capable of recognizing a site in each half of MobA. However, the two fragments of MobA share no regions having similar sequence. Therefore, the recognition of these two fragments of MobA by MobB is directed by something other than sequence, perhaps by a structure common to

both MobA domains. Until the crystal structure of the primase domain of MobA is solved, this idea will be difficult to test.

4.3 EITHER TRANSPORT SIGNAL CAN BE UTILIZED DURING PLASMID TRANSFER

The presence of two transport signals in MobA raises the questions: 1) are the components of these two signals interchangeable, and 2) how are these two signals utilized during plasmid transfer? To answer the first question, the R-site of the relaxase signal was fused to the primase fragment and, conversely, the P-site of the primase signal was fused to the relaxase fragment (Figure 4.5, lines 2 and 3). In both cases, these proteins were unable to transport Cre, even in the presence of MobB, indicating that the R- and P-sites require their respective domains (relaxase or primase) to be active. These data suggest structures of the catalytic domains are important for transport as well as enzymatic activity.

To gain a clearer understanding of how these signals are utilized during plasmid transfer, I measured plasmid transfer as well as Cre transport when either or both of the signaling sites (R-site and P-site) were missing. Since the Cre transport data are presented as a percentage of R751 transconjugants, the plasmid transfer data will be similarly presented. R1162 derivatives containing different internal deletions were mobilized into C600 Nal^R from donors containing R751 and pUT221 when necessary, to supply MobB in *trans*. Transfer frequencies of these plasmids were calculated as transconjugants/donor. Under the same conditions, R751 has a transfer frequency of 0.15 transconjugants/donor. Using this information, transfer frequencies of the plasmids containing deletions in *mobA* were expressed as a percentage of the number of R751 transconjugants. Twenty percent of R751 transconjugants also received R1162 (Figure 4.5, line 1). This percentage of plasmid transfer is much higher than the percentage of Cre recombination (0.097%). This is probably due in part because transfer of a single

nucleoprotein complex could give rise to a transconjugant, while Cre recombination requires the transport of at least four Cre molecules that must then assemble at the Cre sensor on the recipient cell's chromosome.

When the R-site and P-site were individually deleted (Figure 4.5, lines 4 and 5), the frequency of plasmid transfer was low compared to a plasmid containing full-length MobA, but transfer was still detected. However, removing both sites abolished both plasmid transfer and protein transport (Figure 4.5, line 6). In contrast, the Cre fusion in which the P-site was deleted did not show any detectable transport while the fusion with the R-site deletion was transported well (Figure 4.5, lines 4 and 5). A western blot showed that these Cre fusions were expressed in the donor in similar amounts (Figure 4.4, lanes 10 and 11), so the lack of transport of the Cre fusion missing the P-site cannot be explained by degradation of the fusion proteins. The reduced amount of plasmid transfer in the absence of the P-site might result from reduced nicking by MobA rather than inactivation of a transport signal. Why is the R-site able to promote plasmid transfer, but not detectable transport of the Cre fusion? Perhaps the relaxase transport signal is exposed only after binding or nicking *oriT*. If this is true, then in the absence of *oriT* this signal might remain inaccessible and, therefore, unable to facilitate Cre transport. In the case of Cre fused to the relaxase fragment (Figure 4.3, line 11) the signal might have been activated in the absence of *oriT* because the C-terminal half of MobA was missing, thereby altering the structure of MobA slightly. Regardless of the mechanism for activating the relaxase transport signal, it is clear that either signal, relaxase or primase, can be utilized during plasmid transfer.

4.4 THE STRUCTURE OF MOBA IS IMPORTANT FOR TRANSPORT

As mentioned above, the R-site and P-site represent only a portion of the signals required to transport the relaxase and primase fragments, respectively, through the R751

pore. Furthermore, at least some part of the respective catalytic domains is required (Figure 4.3, lines 8 versus 9 and 11 versus 10). What properties of the catalytic domains contribute to the transport signals? Since the relaxase and primase fragments do not share any sequence homology, perhaps there is a structural component to the signal. This notion is supported by in-frame insertions into MobA. R1162-based plasmids containing an insertion of 6 amino acids into the primase domain of MobA between residues 446 and 447 are not transferred (Henderson and Meyer 1999). Data presented in the previous chapter indicated that the primase is not required for transfer (Figure 3.7, E). Thus, the lack of transfer by this plasmid was likely caused by inability to transport through the R751 pore. To test this, the MobA containing this insertion was fused to Cre (Figure 4.6, line 2). No blue transconjugants were observed (<0.0092%). This supports the notion that structure of the MobA primase domain plays a role in signaling transport. Since the full-length MobA fusion is not transported at a high rate (0.097%), the same mutation was introduced into the highly mobile primase fragment (Figure 4.6, line 5). In this case, the insertion had a large effect (Figure 4.6, lines 4 and 5), but did not completely inhibit transport of the fusion protein. Western blot analysis of cleared lysates show that this Cre fusion is stable (Figure 4.4, lane 6), therefore the lowered transport frequency is not due to degradation.

We had already determined that the C-terminus of the primase fragment is not required for transport (Figure 4.3, line 8). With this truncation as a starting point, digestion of pUT1911 by BAL-31 nuclease was used to determine how much of the C-terminal end of the primase fragment could be removed. Interestingly, the largest truncation of this protein that was still capable of transport ended at residue 588, very near the site of the original truncation (residue 599), indicating that the required structural component of the signal was near this region. The sequence of the BAL-31 truncations

revealed that all of the functional truncations contained a pair of arginines located near the C-terminus. Previous papers have suggested that positive charges, often from arginines, are part of the transfer signal in other transport systems (Vergunst et al. 2005; Luo and Isberg 2004), so these arginines could possibly be important in signaling, either because of charge or structure. However, when these arginines in either the truncated or full-length primase domain were changed to a pair of alanines by site-directed mutagenesis (Figure 4.6, lines 6 and 7), the Cre fusion was still transported, although at a rate lower than the parental fusions (Figure 4.6, line 4, and Figure 4.3, line 8, respectively).

Three amino acids were introduced between residues 113 and 114 of the relaxase fragment (Figure 4.6, line 9). Like the insertion into the primase fragment, this insertion also rendered the Cre fusion incapable of transport. Again, a pair of arginines was located at the C-terminal end of this Cre fusion. However, unlike the arginine pair from the fusion of Cre to primase fragment, changing the C-terminal arginines of relaxase fragment to alanines resulted in a lack of detectable transport (Figure 4.6, line 10). Western blot analysis of the donor strain containing this fusion showed that this protein was as stable as the protein lacking this mutation (Figure 4.4, lanes 7 and 8). When this mutation was present in the full-length MobA, transport was unaffected (Figure 4.6, line 3). This is in agreement with the observation that the presence of the primase signal can relieve the need for a relaxase signal (Figure 4.5, line 5). These data would seem to support the idea that positive charges at the C-terminal end is required for transport in the absence of the primase domain. However, when the arginines were changed to lysines (Figure 4.6, line 11), the fusion was still unable to produce blue transconjugants. If positive charges alone were required, then this arginine-to-lysine change should have resulted in successful transport. Perhaps the lysine residues altered the structure of the

relaxase fragment, preventing interaction with MobB, again suggesting that structure can play a role in selection of transported molecules.

Data collected from Cre transport assays have given us some insight into the nature of the MobA transport signal. It is now clear that the MobA transport signal is more complex than those of effector molecules transported by VirB/D4 and Dot/Icm pores. Both domains of MobA contain a transport signal consisting of a non-catalytic site, termed the R-site (relaxase domain) or the P-site (primase domain), as well as a structural component contained within the catalytic region of each domain. The non-catalytic sites are functional only when they are adjacent to their respective domains. In full-length MobA, either signal can be utilized during plasmid transfer, but the primase domain signal appears to be stronger of the two during Cre transport. The presence of MobB during transport greatly increases the frequency of transfer; perhaps by binding the MobA transport signals and facilitating interaction between MobA and the R751 transfer pore.

Chapter 5: Discussion

5.1 REPLACEMENT STRAND SYNTHESIS IN DONOR CELLS

During conjugative transfer, only one strand of the DNA is passed from one cell to another. In the recipient, formation of a transconjugant therefore requires replication of the missing strand. However, the need for strand replacement synthesis in the donor is not immediately obvious because there are multiple copies of this plasmid within the donor cell and the frequency of transfer is much lower than the frequency of replication (Figure 3.1B). The complement to the transferred strand might simply be lost in the donor cell and the missing plasmid replaced by replication of one of the remaining plasmid copies. Test plasmids containing heteroduplex oligonucleotides, differing by a unique restriction site on each strand, were used to determine if a single plasmid molecule could support in the absence of vegetative replication multiple rounds of transfer, which would indicate that replacement strand synthesis occurs in the donor. Plasmid DNA isolated from transconjugant populations arising from matings with donors lacking Rep proteins showed that single molecules of R1162 could undergo multiple rounds of transfer (Figure 3.1B). However, the observed frequency of second-round transfer was lower than the frequency of first-round transfer. This could result from a lag between successive rounds of transfer. Newly formed plasmids in the recipient cell, derived from first round and second round transfer, would be incompatible. Therefore, the low levels of detected second-round transfer in the absence of Rep proteins could be caused by a delay in transfer of the second-round, allowing the first-round strand to form multiple copies in the recipient. Incompatibility would then make it difficult for plasmids arising from second-round transfer to become established.

When the Rep proteins are present, the single test plasmid is quickly amplified by vegetative replication, resulting in multiple plasmid molecules, each capable of transfer to the recipient. Replication of the test plasmid containing the mismatch oligonucleotide would result in NheI and FspI plasmids in the donor cell. Transfer of these replicated plasmids would result in both NheI and FspI plasmids in the recipient, thereby giving either type a chance to become dominant. In the Rep+ donors, rapid vegetative replication of the test plasmid means that neither type of plasmid (NheI or FspI) has an advantage in becoming the dominant plasmid in transconjugants (Figure 3.2B).

Two possibilities were considered to explain the lag period between rounds of transfer in the absence of Rep proteins: either strand replacement in the donor is inefficient, resulting in long intervals between rounds of transfer, or strand replacement in the donor is efficient, but the newly synthesized strand is not immediately available for transfer. When a test plasmid was used where the first round of transfer is unable to establish in the recipient, thus removing the barrier of incompatibility, the transfer efficiency of first- and second-round transfer were found to be similar (Figure 3.3). This indicates that strand replacement in the donor is efficient, but the new strand is not immediately transferred to the recipient cell.

Strand regeneration is efficient, but there is an eclipse period, a significant time interval between rounds of transfer, which might reflect the dissociation of the plasmid from the R751 pore after transfer has occurred. During this eclipse period the R751 pore can select another plasmid molecule for transfer. Thus, when vegetative replication increases the number of plasmid molecules in the donor, the eclipse period between rounds of transfer is not noticeable since the R751 pore has additional plasmids to choose from in the donor. This supply of plasmid molecules at the transfer pore

results in transfer of strands from multiple plasmids without the need to pause for strand regeneration. Rapid selection of different plasmids for transfer raises the question: how is the R751 pore able to capture these plasmids quickly when there are only 10-20 copies per cell? One possibility is that the plasmid population is not randomly distributed within the cytoplasm of the cell. This is supported by earlier work indicating that the R1162 molecules are handcuffed through interaction between relaxosomes (Zhang, Zhang, and Meyer 2003). This linkage of molecules might result in a localized, high density of plasmids within the cell. Another factor preventing random distribution of the plasmids in the cytoplasm is that the plasmids might be targeted to the membrane through interaction with MobB. This possible role for MobB will be discussed in more detail in a later section, but localization of the plasmids to the membrane might increase the probability of the relaxosome interacting with TraG, the coupling protein in the R751 pore. One final possibility that must be considered is that there are multiple pores available to transfer plasmid DNA. There has been is no data indicating that each mating pair share a single pore, and selection of plasmids by multiple pores would increase the rate of transfer of DNA into the recipient cell.

Priming at *oriL* is one possible mechanism for strand regeneration in the donor (Figure 5.1, top). Multiple rounds of transfer from a single molecule were also observed for a test plasmid lacking *oriL*, indicating an *oriL*-independent mechanism of strand replacement (Figure 3.4). However, the frequency of second-round transfer is greater when *oriL* is present, presumably due to strand replacement initiated by the R1162 primase. An increase in second-round transfer was also observed when a primosome assembly site was present on the complement to the transferred strand, indicating that strand replacement can be initiated by priming at the PAS sequence. Recruitment of

R1162 primase to initiate strand replacement makes sense because it would be able to recognize *oriL* exposed by mobilization of the complementary strand.

The *oriL*-independent mechanism for strand replacement might be extension of the 3'-end of the nicked molecule, similar to rolling circle replication (Figure 5.1, bottom). One study reported evidence of strand extension during transfer of an R1162-based plasmid containing two *oriT*s (Erickson and Meyer 1993). Mutations in *oriT* were used to increase the chance of observing greater-than-unit-length, transferred molecules arising from rolling circle replication. However, under these conditions the frequency of transfer was decreased by over 3000-fold, so rolling circle replication is unlikely to account for a majority of strand replacement. The exposed 3'-end of the strand to be replaced might be available for extension for only a brief time during transfer. The amount of time it takes for either a strand to be transferred through the R751 pore or DNA replication to be initiated at a free 3'-end is not known but previous studies mapping the *E. coli* K12 chromosome by Hfr transfer revealed that the entire chromosome (4.6 Megabases) can be transferred in 90 minutes (Taylor and Trotter 1967). This means that DNA can be transferred through the F pore at a rate of approximately 850 bases/second. As a result, there is only a short window of time (about 10 seconds) to initiate strand replacement while the 3'end of the transferring strand is still associated with the template strand. Once initiated, however, it would take DNA polymerase, polymerizing at a rate of 500 bases per second (Burgers and Kornberg 1982), about 12 seconds to replace the missing strand of the test plasmid.

Although either rolling circle replication or priming at *oriL* might be used to initiate strand replacement in order to "reset" the plasmid so that another round of transfer can be initiated from this molecule, the presence of an eclipse period suggests that a plasmid undergoing strand replacement is not immediately available for a

subsequent round of transfer. In the case of the *oriL*-dependent mechanism, this lag might represent the initiation step of strand replacement. The time it takes DNA polymerase III holoenzyme to synthesize the missing strand of a single-stranded template can be measured in seconds (Burgers and Kornberg 1982, 1982). Thus it is doubtful that this step of strand replacement is responsible for the lag period. Perhaps the lag is because the plasmid is inefficiently primed by R1162 primase. If this is the case, then the pore would complete the first round of transfer and release the plasmid before priming occurs. The eclipse is also seen during *oriL*-independent strand replacement, a mechanism that does not require priming by R1162 primase. This indicates that even if strand replacement is, in this case, initiated by extension of the exposed 3'-end, the resulting strand is either not transferred immediately or is not rescued in the recipient. This argues against the idea that multiple copies of the plasmid are rapidly transferred in the form of a concatemer, which MobA then converts to monomers. Plasmids undergoing strand replacement initiated by either an *oriL*-dependent or an *oriL*-independent mechanism probably disassociate from the R751 pore while the replacement strand is being synthesized. During this time, the pore can select another plasmid, thereby ensuring a high rate of transfer into the recipient cell.

5.2 REPLACEMENT STRAND SYNTHESIS IN RECIPIENT CELLS

While *oriL* had been shown to contribute to strand replacement synthesis in the donor, the role of MobA-linked primase in strand replacement in the recipient remained undefined. Previous studies indicated that MobA-linked primase is required for formation of transconjugants, presumably to initiate strand replacement in the recipient (Henderson and Meyer 1999). If this were the case, *oriR*, located on the transferred strand, would be the most likely initiation site. This is supported by the observation that the correctly oriented priming site must be present for formation of transconjugants when

the rate of transfer is low, and therefore when an efficient mechanism for plasmid recovery in the recipient is required (Henderson and Meyer 1996). When *oriR* was deleted from test plasmids used in the present study, however, transconjugants were still observed (Table 3.2), indicating an *oriR*-independent mechanism of strand replacement in the recipient. After the transferred strand arrives in the recipient cell, there is no exposed 3'-end suitable for initiating replacement of the missing strand, so it is reasonable to assume that the *oriR*-independent mechanism involves priming as one of the initiating steps, even if this event occurs at a site other than *oriR*.

Although *oriR* was not required in the recipient, the primase still appeared to be required for transfer, even when a primosome assembly site (PAS) was supplied (Table 3.3). A PAS sequence can substitute for a priming site during strand replacement (Figure 3.4) and should allow strand replacement in the recipient in the absence of the R1162 primase. Therefore, the lack of transconjugants indicates that the requirement for primase is unrelated to strand replacement in the recipient. One possibility is that the primase-inactivating mutation used in the earlier studies caused the protein to misfold in such a way that transfer is unable to proceed in a normal manner. When the pSC101 MobA, a relaxase that does not contain a linked primase, was used instead of R1162 MobA, there was still strand replacement in the recipient (Table 3.4). This result is a strong indication of an *oriR*-independent mechanism for strand replacement in the recipient.

What is the nature of this mechanism? Two possibilities were considered: 1) the TraC primase from R751 was acting on R1162 in the absence the R1162 primase; or 2) the recipient cell encoded a mechanism capable of initiating strand replacement. The TraC primase of the IncP plasmid RP4 is transferred from donor to recipient during conjugation (Rees and Wilkins 1990) where it is presumed to aid in strand regeneration.

TraC is also able to suppress a DnaG mutation in *E. coli* (Lanka et al. 1985), and was able to compliment an *oriC*-deficient M13 mutant (Figure 3.9), indicating that TraC is capable of initiating DNA synthesis on substrates other than R751. However, the presence of R751 TraC in donor cells used in the electroporation/mating assay did not increase the number of transconjugants (Table 3.5). In fact, R751 TraC inhibited transfer of pUT1735 into either *E. coli* or *Salmonella*, possibly due to competition between TraC and the R1162 relaxosome at the R751 pore (Figure 3.6). The R751 primase might be unable to recognize a suitable priming site on R1162 despite its relaxed specificity on other substrates, or the single-stranded DNA molecule might not remain in the recipient long enough for the primase to act upon it. Regardless of the reason, it is clear that the TraC primase is not responsible for *oriR*-independent strand replacement in the recipient.

oriR-directed strand replacement might be important in a recipient in which the host-encoded priming mechanism is less efficient. Work done by Lanka and Barth indicated that TraC primase is important for transfer of RP4 to *Salmonella*, but not *E. coli* (Lanka and Barth 1981). They suggested that this was due to an inefficient mechanism for strand replacement in *Salmonella*. In agreement with that study, deletion of either the MobA-linked primase (Figure 3.7) or *oriR* (Figure 3.8) resulted in few transconjugants in *Salmonella*. When R1162 primase was expressed in *Salmonella* recipient cells, the number of transconjugants increased, but only by 3-fold (Table 3.6). This low level of complementation might reflect an inability of the primase to locate quickly the incoming, single-stranded substrate. Under normal conditions, covalently-linked MobA remains near the origin of replication during transfer. In addition, if primase were transported to the recipient cell during mating, then the single-stranded substrate and the primase would be localized in the same area within the recipient as they

exit the transfer pore. These observations indicated that in the absence of an efficient host encoded mechanism, the R1162 primase and *oriR* become more important for the formation of transconjugants.

What is the host-encoded mechanism responsible for *oriR*-independent transconjugant formation in *E. coli*? One possibility is that strand replacement is being initiated by DnaG primase. This primase is capable of initiating strand replacement after transfer of plasmid ColIb, a plasmid unrelated to R751, in the absence of the plasmid primase, Sog (Chatfield et al. 1982). However, expression of *E. coli* DnaG in *Salmonella* recipients resulted in only a 4-fold increase in the number of transconjugants (Table 3.7). This small increase is similar to the increase observed when unlinked primase (RepB') is expressed in *Salmonella* recipients (Table 3.6). In both cases, the lack of complete complementation might have resulted from the inability of the primase, either DnaG or RepB', to find the single-stranded DNA molecule as it enters the recipient cell. Based on pairwise, global alignment (Needleman and Wunsch 1970) the DnaG primases of *E. coli* and *Salmonella* are 86.2% identical and 92.4% similar. *Salmonella* DnaG is also functional as a primase in *E. coli* (Maurer et al. 1984; Rowen, Kobori, and Scherer 1982). However, *Salmonella* DnaG was unable to complement the *dnaG2903* mutation [originally isolated as *dnaP18* (Murakami et al. 1985)], a mutation that can be complemented by wild-type *E. coli* DnaG (Wada and Yura 1974). This is an indication that, despite their similarities, there are differences between these two primases, and therefore they might behave differently when presented with single-stranded DNA during conjugation.

DnaG is not the only possibility for host-encoded strand replacement. PriA, a protein involved in reinitiation of chromosome replication at stalled forks (Zavitz and Marians 1991), might recognize the single strand in the recipient cell. PriA is also

responsible for directing the formation of primosomes at the PAS sequences of bacteriophage ϕX174 (Wickner and Hurwitz 1975) and plasmid pBR322 (Minden and Marians 1985). In both cases, a hairpin structure is recognized by PriA, which then proceeds to direct the loading of additional replication proteins. A PAS sequence from pBR322 was able to substitute for *oriR* during both vegetative replication and plasmid transfer (Table 3.4) indicating that PriA is able initiate DNA synthesis on transferred strands. Although no PAS sequences, cryptic or otherwise, have been found in R1162, perhaps PriA is recognizing a region of the transferred strand that is displaying a high degree of secondary structure.

A third possible mechanism for host-encoded strand replacement in the recipient involves RNA polymerase (RNAP). Minus strand synthesis of the filamentous phage M13 requires an RNA primer supplied by RNAP with a σ^{70} subunit (Kaguni and Kornberg 1982). Secondary structure again plays a role in directing the initiation of strand synthesis, this time by mimicking the -10/-35 hairpin structure of a promoter (Higashitani, Higashitani, and Horiuchi 1997). Lagging strand synthesis of plasmids that replicate by rolling circle replication is often primed by RNAP (del Solar et al. 1998). Lagging strand synthesis of these plasmids and conjugative strand replacement both utilize a single-stranded molecule as template DNA. The parallels between these two substrates make the idea of initiation by RNAP very attractive. Evidence of interaction between transferred, single-stranded DNA and RNAP has been reported for the plasmid ColIbP-9 (Althorpe et al. 1999). Two genes encoded by this plasmid, *ardA* and *psiB*, are transcribed shortly after arrival of the transferred strand in the recipient cell, before strand replacement occurs. Perhaps after transfer of R1162, RNAP recognizes this molecule in a similar way and polymerizes an RNA primer that can then be extended by DNA polymerase, thereby replacing the missing DNA strand. With the exception of

mobC, all of the genes encoded by R1162 are in the correct orientation to potentially be transcribed in the same manner as *ardA* and *psiB* (Figure 1.1). However, no part of R1162 has yet been identified that can initiate an RNA primer for the purpose of DNA synthesis.

5.3 WHAT MAKES UP THE MOBA TRANSPORT SIGNAL?

Most of the research to date on the secretion of molecules by type IV transfer has focused on the export of effector molecules from bacteria to eukaryotic cells. In particular, the VirB/D4 system of *Agrobacterium* and the Dot/Icm system of *Legionella* have been studied extensively (Luo and Isberg 2004; Vergunst et al. 2005). Recently, researchers have begun to determine the nature of the signals required to target a protein for transfer. Effectors transported by the VirB/D4 system, such as VirF, VirE2 and VirE3, or by the Dot/Icm system, such as SidC, contain a translocation signal located at the C-terminal end of the effector proteins consisting of an array of positively charged residues, typically arginines (Luo and Isberg 2004; Vergunst et al. 2005). RSF1010 MobA was among the proteins tested by Luo and Isberg (2004) that were successfully transported to recipient cells. In fact, a fusion of Cre to the C-terminal 48 amino acids of MobA can be transported through the VirB/D4 pore (Vergunst et al. 2005). In contrast, the same 48-amino acid fragment cannot promote transfer of Cre by the R751 transport system (Figure 4.3, line 3). The possibility that this fusion is transported to recipient cells, but at a rate too low to be detected by the Cre sensor, has not been ruled out. However, it is clear that a C-terminal truncation of the primase fragment is efficiently transported (Figure 4.3, line 8) indicating that the C-terminal 48 amino acids are not required.

Surprisingly, the N-terminal half (relaxase fragment) and C-terminal half (primase fragment) of MobA can be transferred independently of one another, indicating that both

halves contain their own transfer signals (Figure 4.3). Regions within the two halves were identified, the R-site (amino acids 205-284) and the P-site (amino acids 662-709), that were required for transport of the relaxase and primase fragments, respectively (Figure 4.3), but neither of these regions were capable of promoting transport of Cre on there own, so they represent only part of the transport signal for their respective fragments. This is not the first evidence of a complex signal being utilized during type IV transport. The Bep proteins (*Bartonella*-translocated effector proteins) of *Bartonella henselae* require an internal domain, in addition to the signal located in the C-terminal end, for translocation through the *Bartonella* VirB/D4 system into mammalian cells (Schulein et al. 2005). Similarly, the CagA effector molecule of *Helicobacter pylori* requires the N-terminus, as well as the C-terminus, for transfer into mammalian cells (Hohlfeld et al. 2006). Taken together, these are clear evidence that not all type IV secretion signals are as simple as those used by *Agrobacteria*, and that there are a variety of determinants depending on the type IV transport apparatus. However, in addition to any internal signals, all of the proteins described above require the C-terminus of the protein for transport.

The two signals of MobA might have developed during a time when the two domains were expressed as separate peptides. If the relaxase domain was expressed without RepB' primase fused at the C-terminal end, similar to pSC101 MobA, then this domain would be expected to contain a transport signal. When the relaxase and primase genes were expressed as a fusion, the primase domain might have acquired a second signal to aid in transport of this larger protein. Interestingly, although either signal is sufficient for plasmid transfer, the presence of the relaxase signal, alone, is incapable of promoting detectable transport of Cre (Figure 4.5, line 4), suggesting that the primase signal is the stronger of the two. This difference between plasmid transfer and protein

transport might be because protein transport was measured in the absence of *oriT*. Perhaps the relaxase signal of the full-length MobA is exposed only after nicking *oriT*. One can imagine that selecting MobA molecules at random might be an inefficient method of targeting plasmids for transfer. Therefore, exposure of the relaxase signal after nicking might serve to increase the efficiency of plasmid transfer by increasing the likelihood that a DNA-bound MobA is selected for transfer rather than an unbound MobA.

What is the nature of the relaxase and primase signals? There is no obvious similarity between the sequences of the R-site and P-site that would suggest a clear-cut signal. As mentioned above, these sites are not functional as signals when expressed without their respective catalytic (relaxase and primase) domains. This indicates that there is some feature in the relaxase and primase domains that, along with the R-site and P-site, make up the transport signal. Both fragments contain arginines at their C-terminal ends, a feature believed to be important in type IV signal sequences (Vergunst et al. 2005), but loss of the arginines from the C-terminal end of the primase fragment reduced transfer only 1.6-fold (Figure 4.3, line 8 vs. Figure 4.6, line 7). There are, however, additional groups of arginines throughout the primase region, any one of which might be acting as a signal. Transport of the relaxase fragment was inhibited when the C-terminal arginines where changed to alanines (Figure 4.6, line 10), which would seem to agree with the recent literature. However, replacing the arginines with lysines, an amino acid that would also be positively charged within the cytoplasm of the cell, also inhibited transport (Figure 4.6, line 11), indicating that positive charges alone are insufficient.

Perhaps the effect of these amino acid substitutions on transport is caused by an alteration in the structure of the domain as opposed to changes in charge. In agreement

with this possibility, internal insertions of amino acids into either the relaxase or primase domain results in reduced transport (Figure 4.6, lines 2, 5 and 9). Insertion of six amino acids into the primase fragment resulted in a reduction in transport greater than 600-fold. Likewise, insertion of only three amino acids into the relaxase fragment was enough to prevent any detectable transport. It is important to note that although the Cre transport assay does not require nicking or priming of DNA, alterations to these domains did negatively affect transport. This is a strong indication that the structure within these two domains is important for transport.

Although the crystal structure of the relaxase domain of MobA has been solved (Monzingo et al. 2006), the primase domain has not, making a direct comparison of the secondary structure of these two domains impossible. Threading analysis (Guex and Peitsch 1997) of the two domains using the relaxase domain as a template revealed a small region in both domains that that were 21% identical (Figure 5.2). The two regions had six amino acids in common with similar spacing between each residue. In addition, these two regions are very near the sites where the amino acid insertions discussed above were located. Perhaps alteration of these structures by amino acid insertion is linked to the reduction in transport. While this type of analysis is not conclusive, it does present the possibility that both of these domains contain signature structures that are being recognized as part of the transport signal.

5.4 THE ROLE OF MOBB IN TRANSPORT OF MOBA

One of the most striking results from the Cre transfer assay is that the MobB accessory protein is required (Figure 4.2). The role of this protein in transfer, aside from its ability to stimulate nicking of the transfer strand, has been suggested in previous experiments (Perwez and Meyer 1999) but never directly demonstrated. The relaxase and primase fragments both require MobB for efficient transport, but there are no similarities

in the amino acid sequences of these domains that would suggest a binding site common to both fragments. Perhaps MobB is recognizing a structural feature of these fragments, possibly the transport signal described above.

Little is know about the structure of MobB. Analysis of the secondary structure of MobB, predicted by the program PHDhtm (Rost, Fariselli, and Casadio 1996), suggests that MobB is a membrane protein with an N-terminal cytosolic domain consisting mainly of alpha helices. Overexpression of MobB is toxic to *E. coli*, consistent with the prediction that MobB is inserted in the membrane (unpublished results). It would be interesting to see if the transmembrane domain of MobB is required for stimulating the nicking activity or transport of MobA. Regardless, the presence of a transmembrane domain in MobB might be a clue to its role in MobA transport.

Perhaps MobB targets the relaxosome complex to the membrane (Figure 5.3). This might help position the MobA-DNA complex near the R751 pore, thereby increasing the frequency of transfer. The targeting of substrates, including relaxases, to the transfer pore is a role normally attributed to the TraG coupling protein (Szpirer, Faelen, and Couturier 2000; Cabezon, Sastre, and de la Cruz 1997). However, the coupling protein might have a higher affinity for substrates encoded by the plasmid supplying the transfer pore, placing R1162 MobA at a disadvantage. If this is the case, then targeting MobA to the membrane might help MobA compete with other substrates, such as the R751 relaxase and conjugative primase, by placing MobA and TraG closer together or stabilizing the interaction between the two proteins.

Another possible role for the hydrophobic region of MobB is to act as a chaperone for MobA. The single-stranded DNA-binding protein, VirE2, of *Agrobacterium* is bound and kept in an inactive state by a chaperone, VirE1, prior to type IV transport to

the plant cell (Deng et al. 1999). Chaperones are also common in type III transport (Stebbins and Galan 2001). Secreted effector proteins, such as the Yop proteins of *Yersinia* and the Sip proteins of *Salmonella*, are kept in a non-functional state by chaperones until they are transferred to the target cell by the type III transport system (Bennett and Hughes 2000). However, the possibility that MobB is a chaperone protein raises the question: how would the two roles of MobB, chaperone versus relaxosome stabilization, be regulated in this model? If MobB were unfolding MobA while associated with the relaxosome, MobA would be unable to act as a functional relaxase. Therefore, unfolding of MobA would have to occur after MobA has had a chance to bind and nick *oriT*. MobB might be unable to alter the structure of MobA while the relaxase is tightly bound to *oriT*. Perhaps after nicking, the structure of MobA is altered sufficiently to expose a chaperone-binding site.

The requirement of MobB during transport of MobA represents a novel mechanism to increase the efficiency of R1162 transfer during conjugation. To date, most research on relaxase transport focuses on the interaction between the relaxase and the coupling protein. The research presented here is strong evidence that the relaxasae is not the only protein to interact with the coupling protein during transport. Understanding the mechanism used by MobB to increase the efficiency of MobA transport might give us insight into how R1162 is able to compete for use of conjugative pores encoded by other plasmids.

Tables

Table 2.1 – *E. coli* and *Salmonella* strains used.

E. coli	**Properties**
C600	F- [e14$^-$ (McrA$^-$) or e14$^+$ (McrA$^+$)] *thr*-1 *leuB*6 *thi*-1 *lacY*1 *glnV*44 *rfbD*1 *fhuA*21 (Appleyard 1954)
C600 Nal^R	Nalidixic acid-resistant mutant of C600 (Figurski et al. 1976)
MV10	F- [e14$^-$ (McrA$^-$) or e14$^+$ (McrA$^+$)] *thr*-1 *leuB*6 *thi*-1 *lacY*1 *glnV*44 *rfbD*1 *fhuA*21 Δ*trpE*5 (Hershfield et al. 1974)
MV12	F- [e14$^-$ (McrA$^-$) or e14$^+$ (McrA$^+$)] *thr*-1 *leuB*6 *thi*-1 *lacY*1 *glnV*44 *rfbD*1 *fhuA*21 Δ*trpE*5 *recA*56 (Hershfield et al. 1974)
MV12 Nal^R	Spontaneous nalidixic acid-resistant mutant of MV12
MC1061	F- *araD*139 Δ(*ara-leu*)7696 *galE*15 *galK*16 Δ(*lac*)X74 *rpsL* (Str^r) *hsdR*2 (r_K^- m_K^+) *mcrA mcrB*1 (Wertman, Wyman, and Botstein 1986)
JC9239	F- *recF*143 *thr*-1 *leu*-6 *psoA*2 *lacY*1 *galK*2 *his*-4 *xyl*-5 *mtl*-1 *argE*3 *thi*-1 T_6^r l^s Sm^r (Horii and Clark 1973)
ES568	F- *fhuA*2 *lacY*1 *tsx*-1 or *tsx*-70 *gluV*44(AS) *gal*-6 l^r *xylA*7 *mtlA*2 *mutL*13 (Lieberfarb and Bryson 1970)
JC355	F- *leuB*6 *fhuA*2 *lacY*1 *gluV*44(AS) *gal*-6 l^r *hisG*1(5) *rfbD*1(3) *galP*63 *argG*6 *malT*1 *xylA*7 *mtlA*2 *metB*1 (Clark, Maas, and Low 1969)
Top10::LKL	F- *mccrA* Δ(*mrr-hsdRMS-mcrBC*) f80*lacZ*ΔM15 Δ*lacX*74 *deoR recA*1 *araD*139 Δ(*ara-leu*)7697 *galU galK rpsL*(Strr) *endA*1 *nupG* Ω(312kb::*lacZ*α::(*lox-aphA*-3-*lox*))
JM103	F' *traD*36 *lacI^q* Δ(*lacZ*)M15 *proA$^+$*/*B$^+$*/*endA*1 *glnV sbcBC thi*-1 *rpsL* (Str^r) Δ(*lac-pro*) (P1) ($r_K^+m_K^+$ $r_{P1}^+m_{P1}^+$) (Yanisch-Perron, Vieira, and Messing 1985)
Salmonella	**Properties**
LT2	Prototroph (Zinder and Lederberg 1952)
LT2 Nal^R	Spontaneous nalidixic acid-resistant mutant of LT2
LB5000	*metA*22 *metE*551 *trpD*2 *ilv*-452 *leu*$^-$ *pro*$^-$(leaky) *hsdLT*6 *hsdSA*29 *hsdSB*$^-$ *strA*120 (Bullas and Ryu 1983)

Table 2.2 – Plasmids used to study conjugative strand replacement.

Helper plasmids		
Name	**Properties**	**Construction/source**
pMS94	R1162 Rep+	Meyer, Hinds and Brasch, 1982
pUT1543	R1162 Mob, Pri+, Rep+	Henderson and Meyer, 1999
pUT1559	R1162 Mob, Pri+	Henderson and Meyer, 1999
pUT1790	R1162 Mob, Pri-	Insertion of 18 bases (oligonucleotide 14) at the AflIII site of pUT1559
pUT1798	R1162 Mob, Pri-, Rep+	Insertion of 18 bases (oligonucleotide 14) at the AflIII site of pUT1543
pUT1795	pSC101 Mob	pSC101 (Cohen and Chang 1977) amplified with primers 189 and 190 and ligated into AflIII and EcoRV sites of pUT1628 (Meyer 2000). EcoRV/SalI fragment of this plasmid ligated into same sites of pBR322 (Bolivar et al. 1977)
pUT1786	Pri+	repB' ORF amplified with primers 428 and 429 and ligated into ApaLI and PstI sites of pACYC177 (Chang and Cohen 1978)
pUT1584	MobB+	Henderson and Meyer, 1999
pUT459	Rep+, Pri+	Henderson and Meyer, 1999
pUT1612	Integrase+	Henderson and Meyer, 1999
pUT1836	DnaG+	BamHI fragment from pPL184 (Stamford, Lilley, and Dixon 1992) ligated into BamHI site of pWSK129 (Wang and Kushner 1991)
R751	IncP wild type	(Jobanputra and Datta 1974)
pUT1757	IncP, TraC-	By homologous recombination of a kanamycin resistance gene into the *traC* gene of R751 (see text)
Test plasmids		
Name	**Properties**	**Construction/source**
pUT1557	R1162 oriT, oriL+/oriR+	Henderson and Meyer, 1999
pUT1613	R1162 oriT, oriL+/oriR+, attP	Henderson and Meyer, 1999
pUT1740	R1162 oriT, oriL+/oriR+, attP	ApaI and NgoMIV sites flanking *oriV* introduced via inverse PCR with primers 298 and 299
pUT1735	R1162 oriT, oriL+/oriR+, attP	*oriV* fragment of pUT1101 (Becker, Zhou and Meyer, 1996) introduced into pUT1613

Continued next page

Table 2.2 continued

Test plasmids		
Name	**Properties**	**Construction/source**
pUT1742	R1162 oriT, oriL+/oriR+, attP, pBR322 chimera	pBR322 ligated to pUT1735 at BamHI site
pUT1743	R1162 oriT, oriL-/oriR+, attP, pBR322 chimera	*oriL* site of pUT1742 between EcoO109I and SmaI removed
pUT1750	R1162 oriT, oriL+/oriR-, attP, pBR322 chimera	*oriR* site of pUT1742 between SmaI and EcoRV removed
pUT1773	R1162 oriT, oriL-/oriR-, attP, pBR322 chimera	*oriV* site of pUT1742 between EcoO109I and EcoRV removed
pUT1769	R1162 oriT, oriL+/PAS, attP	pBR322 PAS amplified with primers 397 and 398 and ligated into pUT1735
pUT1815	pSC101 oriT, oriL+/oriR+, attP	Inverse PCR of pUT1735 to replace R1162 *oriT* region with pSC101 oriT region
pUT1827	pSC101 oriT, oriL+/oriR+, attP, pBR322 chimera	MfeI-BstXI fragment of pUT1815 ligated into same sites of pUT1742
pUT1828	pSC101 oriT, oriL-/oriR+, attP, pBR322 chimera	MfeI-BstXI fragment of pUT1815 ligated into same sites of pUT1773

Table 2.3 – Cre fusions used to study MobA transport.

Name	**Protein fused to Cre (amino acid residues)**	**Construction/source**
pUT1881	MobA(2-709)	R1162 amplified with primers 643 and 644
pUT1882	MobA(388-709)	R1162 amplified with primers 644 and 645
pUT1885	Cre only	P1 *cre* amplified with primers 611 and 652, ligated into NheI and SalI of pBR322
pUT1886	MobA(2-296, 351-309)	pUT1371 (Perwez and Meyer, 1999) amplified with primers 643 and 644
pUT1902	MobA(2-204)	R1162 amplified with primers 643 and 654
pUT1903	MobA(324-709)	pUT1530 (Perwez and Meyer, 1999) amplified with primers 643 and 644
pUT1904	MobB	R1162 amplified with primers 657 and 658
pUT1905	MobA(2-284)	R1162 amplified with primers 643 and 656
pUT1908	MobA(2-223, 324-709)	pUT1562 (Perwez and Meyer, 1999) amplified with primers 643 and 644, KpnI fragment ligated into pUT1903
pUT1909	MobA(324-386)	pUT1903 amplified with primers 611 and 663, ligated into NheI/SalI of pBR322
pUT1910	MobA(2-280, 324-709)	pUT1530 amplified with primers 643 and 644, KpnI fragment ligated into pUT1903
pUT1911	MobA(324-599)	BlpI-digested pUT1903 treated with klenow and ligated with oligo 11
pUT1912	MobA(662-709)	AgeI fragment from pSDM3204 (Vergunst, et. al., 2005) ligated into AgeI/NgoMIV of pUT1885
pUT1917	MobA(2-709), MobB-	2-step PCR of R1162 using primers 643 and 674, and 675 and 644
pUT1920	64aa-MobA(2-709)	pACYC177 amplified with primers 678 and 679 and ligated into KpnI site of pUT1881
pUT1921	64aa-MobA(388-709)	pACYC177 amplified with primers 678 and 679 and ligated into KpnI site of pUT1882
pUT1922	MobA(324-386)-Aph	2-step PCR of pUT1903 with primers 649 and 690, and pACYC177 with primers 688 and 689
pUT1930	MobA(2-284)+5aa	R1162 amplified with primers 643 and 700
pUT1933	MobA(2-447(6aa)448-709)	BstXI/BlpI fragment of pUT1790 ligated into pUT1881

Continued next page

Table 2.3 continued

Name	**Protein fused to Cre (amino acid residues)**	**Construction/source**
pUT1935	MobA(324-447(6aa)448-709)	pUT1933 amplified with primers 644 and 701
pUT1938	MobA(324-709), RR587AA	pUT1903 amplified with primers 702 and 707
pUT1939	MobA(2-284)+5aa, RR281AA	pUT1930 amplified with primers 643 and 713
pUT1942	MobA(324-599), RR587AA	BlpI-digested pUT1938 treated with klenow and ligated with oligo 11
pUT1944	MobA(2-284)+5aa, RR281KK	pUT1930 amplified with primers 643 and 719
pUT1947	MobA(2-113(3aa)114-284)+5aa	BstXI-digested pUT1930 treated treated klenow, ligated with oligo 14, digested with XhoI, treated with klenow and ligated
pUT1948	MobA(205-284)+5aa	pUT1930 amplified with primers 700 and 720
pUT1958	MobA(205-284, 388-709)	pUT1930 amplified with primers 720 and 732 and ligated into KpnI site of pUT1882
pUT1959	MobA(2-205, 324-387)	pUT1902 amplified with primers 649 and 731 and ligated into KpnI site of pUT1909
pUT1961	MobA(2-323, 388-709)	pUT1487 amplified with primers 643 and 644
pUT1964	MobA(2-204, 324-709)	R1162 amplified with primers 643 and 736 and ligated into KpnI site of pUT1903
pUT1975	MobA(2-204, 388-709)	Acc65I fragment of pUT1964 ligated into same site of pUT1961
R1162-based plasmids		
Name	**MobA expressed (amino acid residues)**	**Construction/source**
R1162	MobA(1-709)	(Barth and Grinter 1974)
pUT1487	MobA(1-323, 388-709)	(Henderson 1998)
pUT1965	MobA(1-204, 324-709)	BstZ17I-KpnI fragment of pUT1964 ligated into same sites of pUT1344 (Henderson 1998)
pUT1970	MobA(1-204, 388-709)	EcoO109I-KpnI fragment of pUT1965 ligated into same sites of pUT1487

Table 2.4 – List of oligonucleotidess used.

Name	Sequence
11	CTAGTCTAGACTAG
14	CTCGAGGCCTCGAG
189	GCACGTTCAGACGTGTCAGCG
190	GCGCGGGATATCCAATTGCGCCCTTCAGC
258	AGATCTGTGCGCTAGCTCGGCCGG
259	AATTCCGGCCGAGCTTGCGCACAGATCTAGCT
298	GATAATCATGGATGGATCCTTCCAACACCCCGCC
299	TAAGAATAATCCGCTAGCCGCGGTTATCAAGCGCCC
306	CTAGCGGATTATTCTTAGATAACCATGGATC
307	CTAGCGGATTATTCTTAGATAACCATGGATC
386	GACACCCTGGAATTCGCCTTGCGCAG
387	CGAGGTACCGGCGCGCG
388	CTGCACGCCAGGATCCCGGACAAGC
389	CGATGCCGAGCGGCCTGCAGTG
397	AGTGCACCATAGGCCCTGTGAGATATCGCACAGATGC
398	CCTGCGTTATCCCGGGATTCTGTCCGATAACCGTATTACCG
428	TGAGTACCAACTGCAGCACTACATGCTGAAATCTGGCCCG
429	GGCTCGCGTTGGTGCACCGATGAAGAACGAC
580	CGATAACTTCGTATAATGTATGCTATACGAAGTTATGG
581	GATCCCATAACTTCGTATAGCATACATTATACGAAGTTATCGGTAC
611	AAACCGCTAGCTGAGTGTTAAATGTCCAATTTACTG
630	ATCGCAGTATGATTGATTACCTGACAGCGTGTATTCCTCTAAACCGCCTCTCCCCG
631	ATAGATACCTAAGTTATATTTCTTACTCTGCGGACAGTGAAAACCTCTGACACA
643	ACAGGGAGGCGGTACCGCGATTTATCACCTTACG
644	AGTAGTCATAGTCGACCAGGCGTGAGTACCAACG
645	GCTTCCTGGCGGTACCAAGAACGACAGGACTTTGC
649	CTGGTCTGGACACAGTGCCCGTG
652	TGCAATCGTCGACGCGTTATTGGCTAGGTACCATCTTCCAGC
654	TCGGCGCCAGGTCGACCTAGGGCAG
656	GTCTGGCTCGTCGACCTACGGGCTTCG
657	CGGGAGGCAATAGGGTACCAACGCAATCGACAGAGTG
658	GCAAAGTCCTGTCGACCTTCATCGGGCCACCAAGC
663	GCAAAGTCCTGTCGACCTTCTACGGGCCACCAAG

Continued next page

Table 2.4 continued

Name	Sequence
674	TCCCGGTACTCCTGGAGGTCGATGATC
675	GGAGTACCGGGAGGCCATCGACCACGAACG
678	AATGTAGGTACCGATGAGATGGTCAGACTAAACTG
679	ATGCAAGGTACCCAGGAACACTGCCAG
688	GTTACAACCAGTCGACCAATTCTGATTAGAAAAACTCATCG
689	GCGCTTGGTGGCCCGAGCCATATTCAACG
690	CGTTGAATATGGCTCGGGCCACCAAGCGC
700	AGGCCCTTTCGTCGACAAGAATTCTCATGTTTGACAGCTTATCATCGGGCTTC
701	AGTTGCAGGAGGTACCCGCCGAGAGCAGCGC
702	GTGATAGCAAAGCTGCCGCTCGGGCAGTTCGAGGCTGGCCAGTGCCGCGGCCT
707	AGTTGCAGGAGGTACCCGCCGAGGTGAGCAGCGC
713	CCTTTCGTCGACAAGAATTCTCATGTTTGACAGCTTATCATCGGGCTAGCAGCGCCAGTGTC
719	CCTTTCGTCGACAAGAATTCTCATGTTTGACAGCTTATCATCGGGCTTTTTTTGCCAGTGTC
720	ATCGAGCGCGGTACCGGTGTTCAC
731	TTGGTCGGTACCGGGCAGGCGC
732	ACAGCTGGTACCCGGGCTTCGTCG
736	GTGAACGGTACCCAGGCGCTCGATGC

Table 3.1 – Transfer of test plasmid in the absence of *oriL*.

	Test plasmid	Donors	Recipients	Average Transconjugants/plate
1	oriL+	Rep-	Int-	1
2	oriL-	Rep-	Int-	0.5
3	oriL+	Rep-	Int+	310
4	oriL-	Rep-	Int+	121.5

Table 3.2 – Transfer of test plasmid in the absence of *oriR*.

	Test plasmid	Donors	Recipients	Average Transconjugants/plate
1	oriR+	Rep-, TraC+	Int-	1.4
2	oriR-	Rep-, TraC+	Int-	0.5
3	oriR+	Rep-, TraC+	Int+	431.5
4	oriR-	Rep-, TraC+	Int+	238.7
5	oriR-	Rep-, TraC+	Int+	70.7
6	oriR-	Rep-, TraC-	Int+	279.7

Table 3.3 – Transfer of test plasmids in the absence of primase and priming sites.

	Test plasmid	Donors	Recipients	Average Transconjugants/plate
1	oriL+/oriR+	Rep-, Pri+	Int+	692
2	oriL-/oriR-	Rep-, Pri+	Int+	330
3	oriL+/PAS	Rep-, Pri+	Int+	662.5
4	oriL+/oriR+	Rep-, Pri-	Int+	8.5
5	oriL-/oriR-	Rep-, Pri-	Int+	10.5
6	oriL+/PAS	Rep-, Pri-	Int+	21.5

Table 3.4 – Transfer of pSC101 *oriT* test plasmids by pSC101 or R1162 MobA.

	Test plasmid	Donors	Recipients	Average Transconjugants/plate
1	R1162 oriT	R1162 MobA	Int+	707
2	pSC101 oriT	R1162 MobA	Int+	1490
3	R1162 oriT	pSC101 MobA	Int+	0
4	pSC101 oriT	pSC101 MobA	Int+	333

Table 3.5 – Transfer of pSC101 *oriT* test plasmids in the absence of TraC and priming sites.

	Test plasmid	Donors	Recipients	Average Transconjugants/plate
1	oriL+/oriR+	Rep-, TraC+	Int+	281
2	oriL-/oriR-	Rep-, TraC+	Int+	297
3	oriL+/oriR+	Rep-, TraC-	Int+	1049
4	oriL-/oriR-	Rep-, TraC-	Int+	1103
5	oriL+/oriR+	Rep-, TraC+	Int+, Pri+	251
6	oriL-/oriL-	Rep-, TraC+	Int+, Pri+	319

Table 3.6 – RepB'-directed replacement strand synthesis in the recipient.

	Strain	Recipients	Average Transconjugants/plate	Fold increase
1	E. coli	Int+	1274	1.1 ± 0.05
2	E. coli	Int+/Pri+	1406	
3	Salmonella	Int+	31	3.3 ± 0.7
4	Salmonella	Int+/Pri+	102	

Table 3.7 – DnaG-directed replacement strand synthesis in the recipient.

	Strain	Recipients	Average Transconjugants/plate	Fold increase
1	E. coli	Int+	2260	1.5 ± 0.005
2	E. coli	Int+/DnaG+	3290	
3	Salmonella	Int+	25.5	3.9 ± 0.8
4	Salmonella	Int+/DnaG+	88.7	

Figures

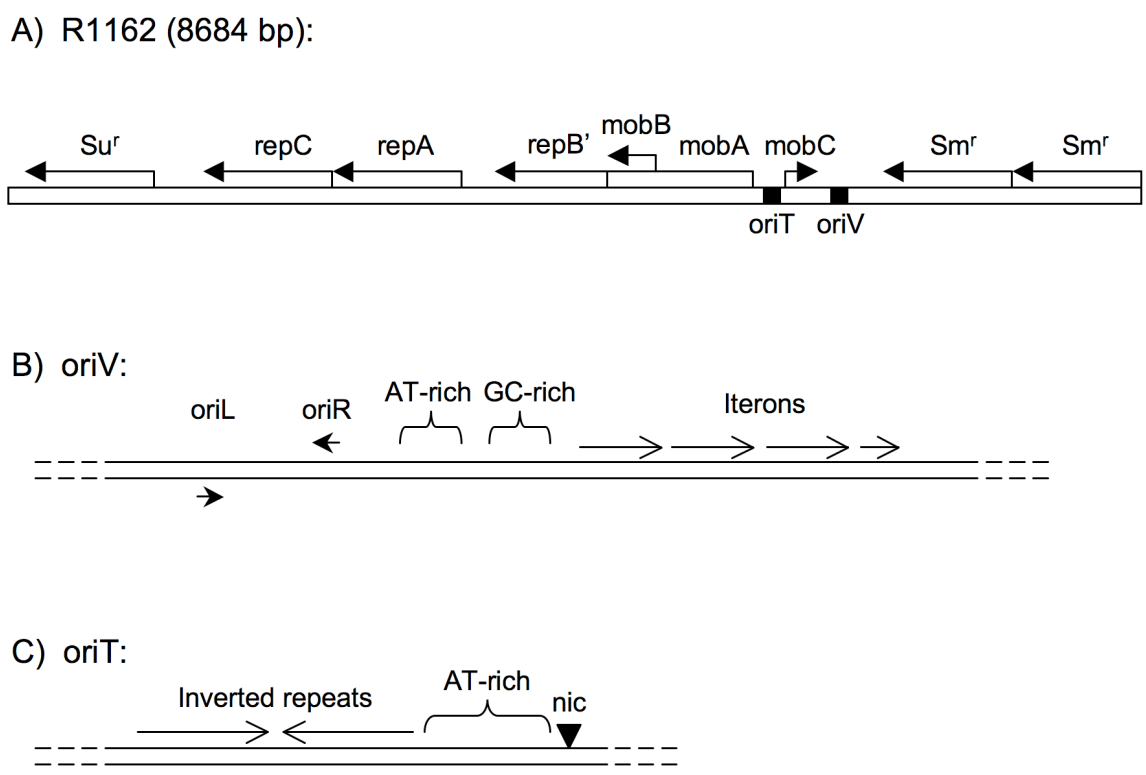


Figure 1.1 – Map of plasmid R1162 and detail of *oriV* and *oriT*.

A) Open reading frames are represented as bent arrows with the vertical portion denoting the start site. B) Single stranded priming sites are denoted by short arrows, indicating the direction of DNA synthesis after initiation. The location of the three-and-a-half direct repeats (iterons) are also shown. C) The nicking site (nic) is marked by an inverted triangle. The inverted repeats are also shown.

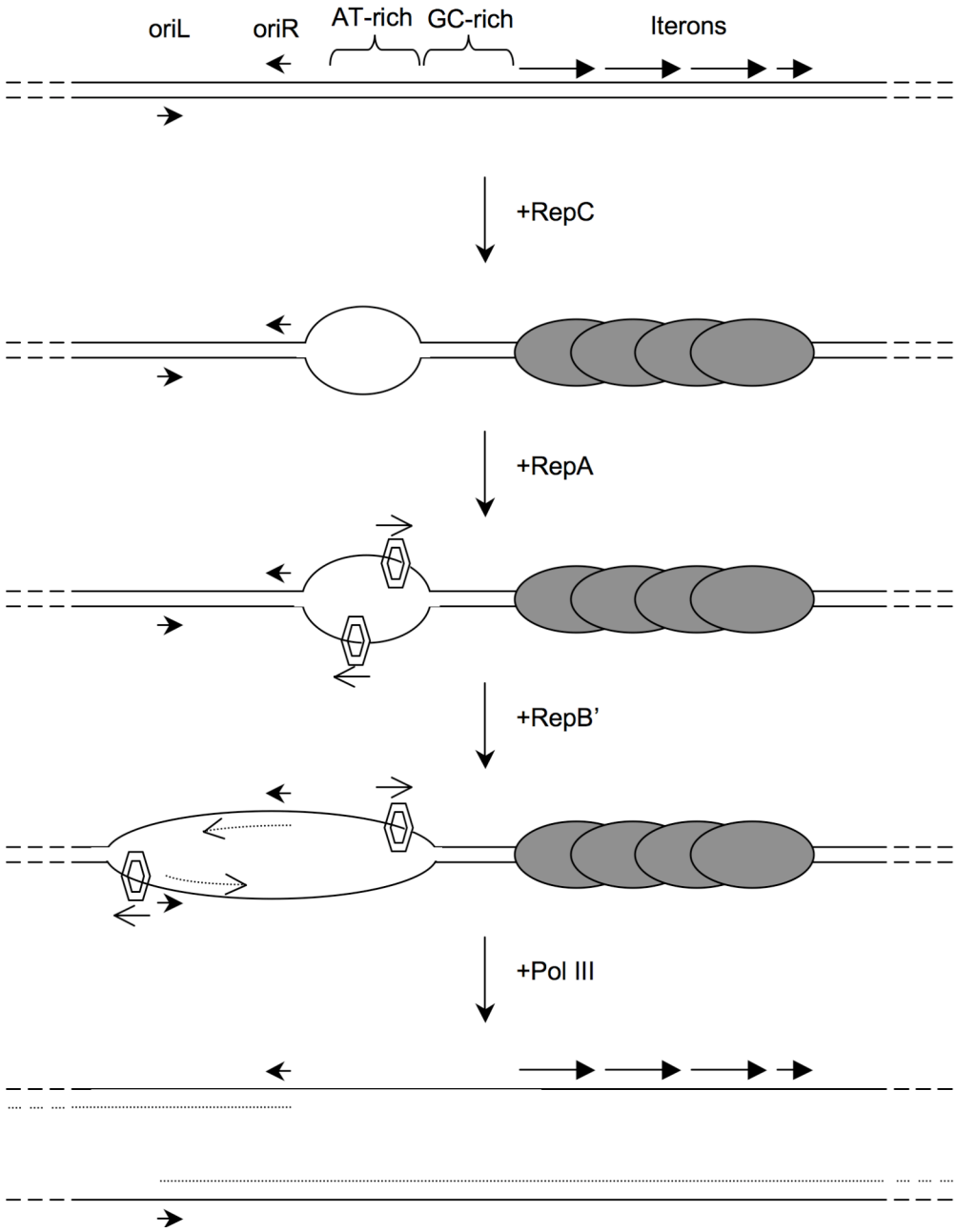


Figure 1.2 – *oriV*-directed replication of R1162 DNA.

Illustration of replication at the R1162 *oriV*. RepC binds the iterons and distorts the helical nature of the DNA at the AT-rich region, allowing RepA access. RepA unwinds the DNA further exposing the priming sites *oriL* and *oriR*. RepB' primes the single stranded priming sites and host-encoded polymerase III extends the primers in opposite directions.

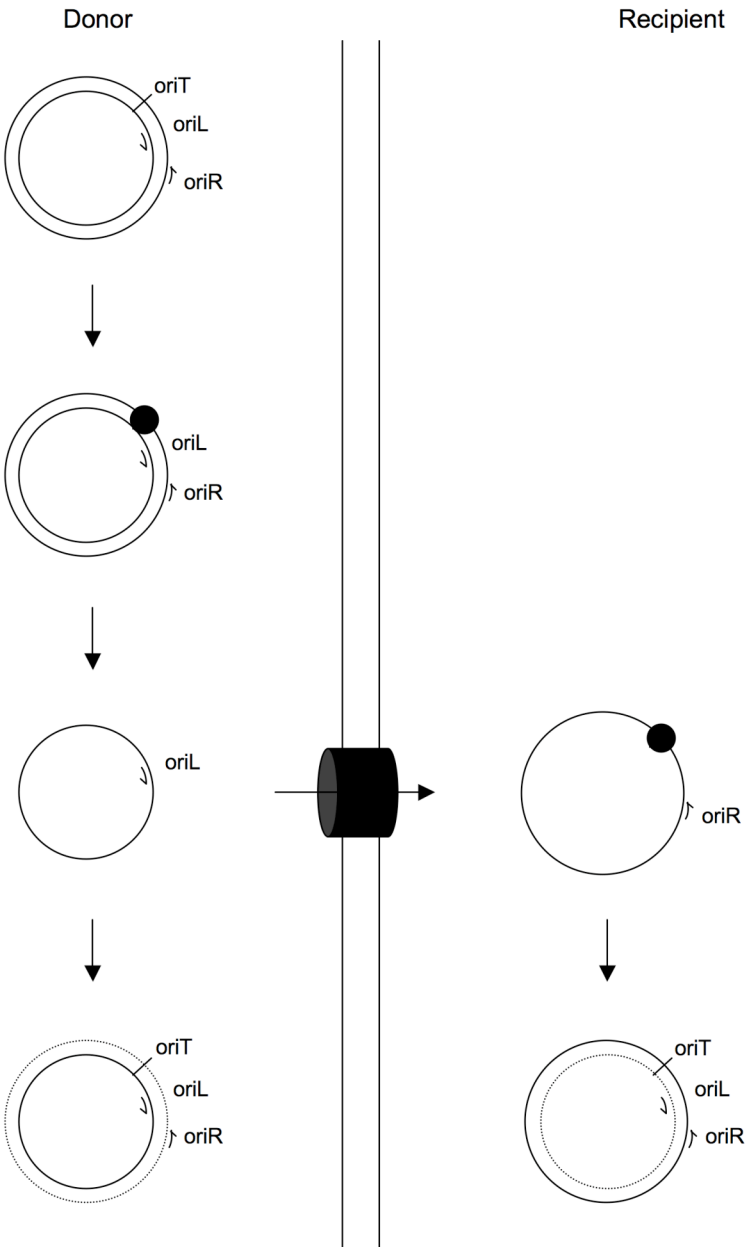


Figure 1.3 – Orientation of priming sites during onjugative transfer.

R1162 is transferred through the IncP transfer pore after MobA (black circle) binds and nicks the *oriT*. The positions of the priming sites (arrows) are shown in relation to *oriT*.

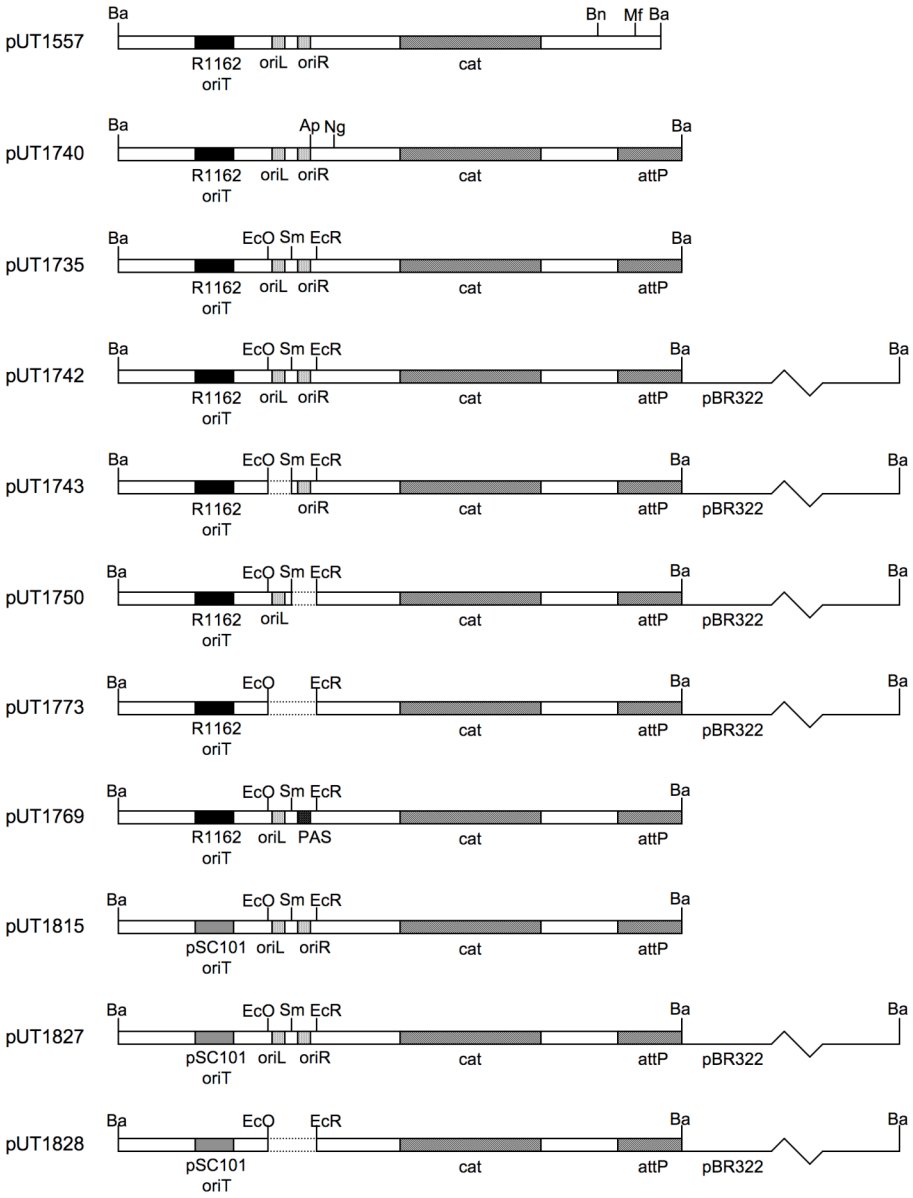


Figure 2.1 – Plasmids used in electroporation/mating assays

All test plasmids contained an *oriT* (either R1162 or pSC101) and a gene responsible for chloramphenicol resistance (*cat*). The *attP* site was used for integration into the host chromosome. ApaI (Ap), BamHI (Ba), BanII (Bn), EcoO109I (EcO), EcoRV (EcR), MfeI (Mf), NgoMIV (Ng), SmaI (Sm)

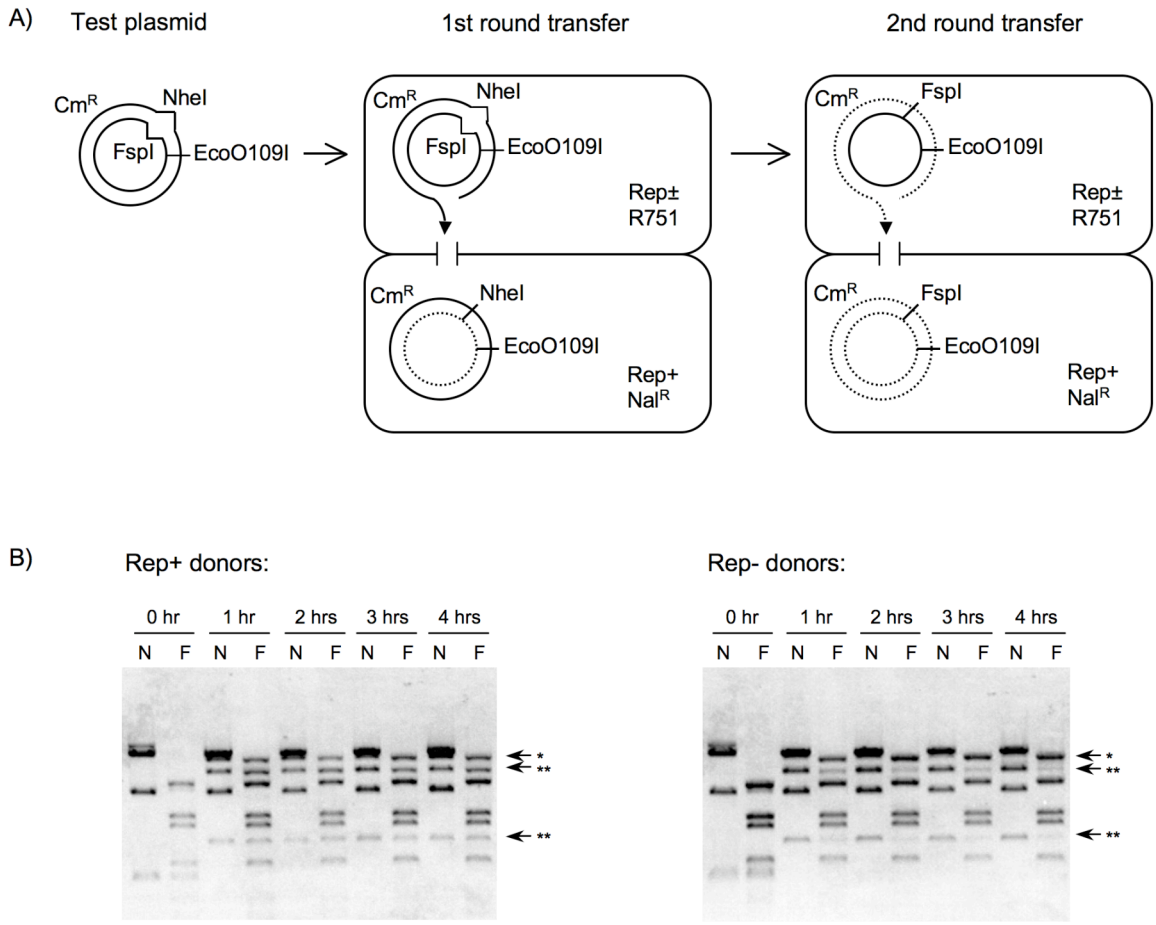
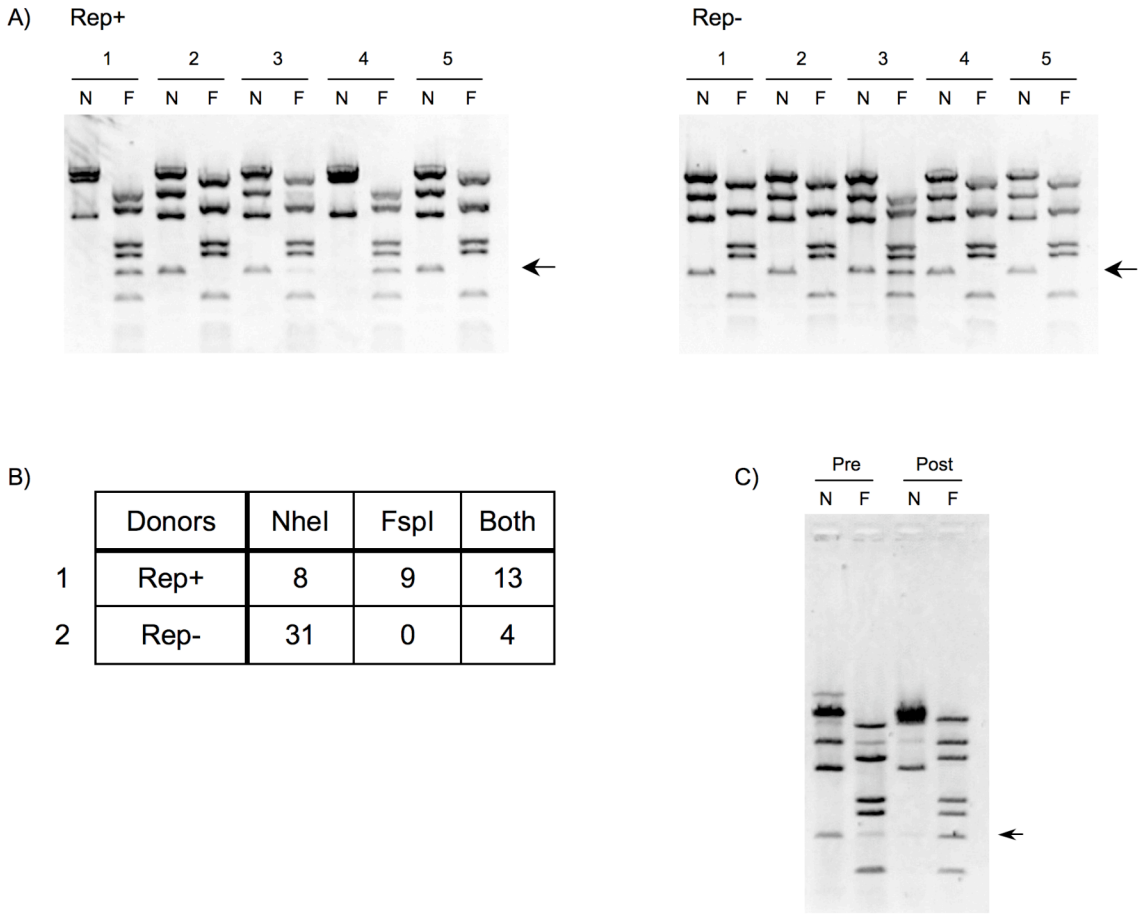


Figure 3.1 – Distribution of multiple rounds of transfer in a population.

A) Test plasmid DNA was introduced into Rep- or Rep+ donors and mated with Rep+ recipient cells. First round transfer would result in the formation of an NheI-containing plasmid; second round transfer would result in the formation of an FspI-containing plasmid. B) Plasmid DNA was extracted from groups of 25 colonies at each time point and analyzed by digestion with EcoO109I and either NheI (N) or FspI (F). The single asterisk marks the position of plasmid DNA digested by only EcoO109I. The double asterisks mark the positions of plasmid fragments resulting from digestion with both EcoO109I and the indicated enzyme.



	Donors	NheI	FspI	Both
1	Rep+	8	9	13
2	Rep-	31	0	4

Figure 3.2 – Multiple rounds of transfer from a single molecule.

A) Plasmid DNA from individual transconjugants was isolated and digested with EcoO109I and NheI (N) or EcoO109I and FspI (F). The arrow indicates the expected size of fragments resulting from digestion with both enzymes. B) Number of transconjugants containing NheI plasmids, FspI plasmids or both, after transfer from Rep- or Rep+ donors. C) Enrichment of FspI plasmids after treatment with NheI and phosphatase.

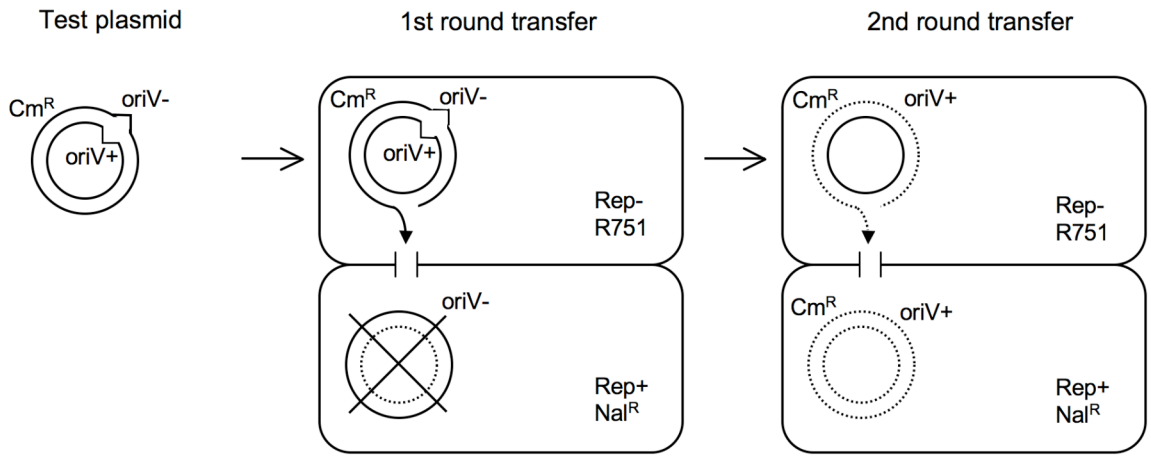


Figure 3.3 – Transfer frequency of first- and second-round transfer.

The NheI/FspI mismatch test plasmid and the oriV-/oriV+ mismatch test plasmid were separately electroporated into Rep- donor cells. The donors were then mated with Rep+ recipient cells and plated on TYE supplemented with chloramphenicol and nalidixic acid to select for transconjugants. In a parallel reaction, NheI/FspI mismatch test plasmids were electroporated into Rep+ donors, mated, and plated on TYE supplemented with chloramphenicol to estimate the number of potential donors. The frequency of first round transfer was calculated as the number of transconjugants from the NheI/FspI mating divided by the estimated number of potential donors, while the frequency of second round transfer was calculated as the number of transconjugants from the oriV-/oriV+ mating divided by the number of potential donors.

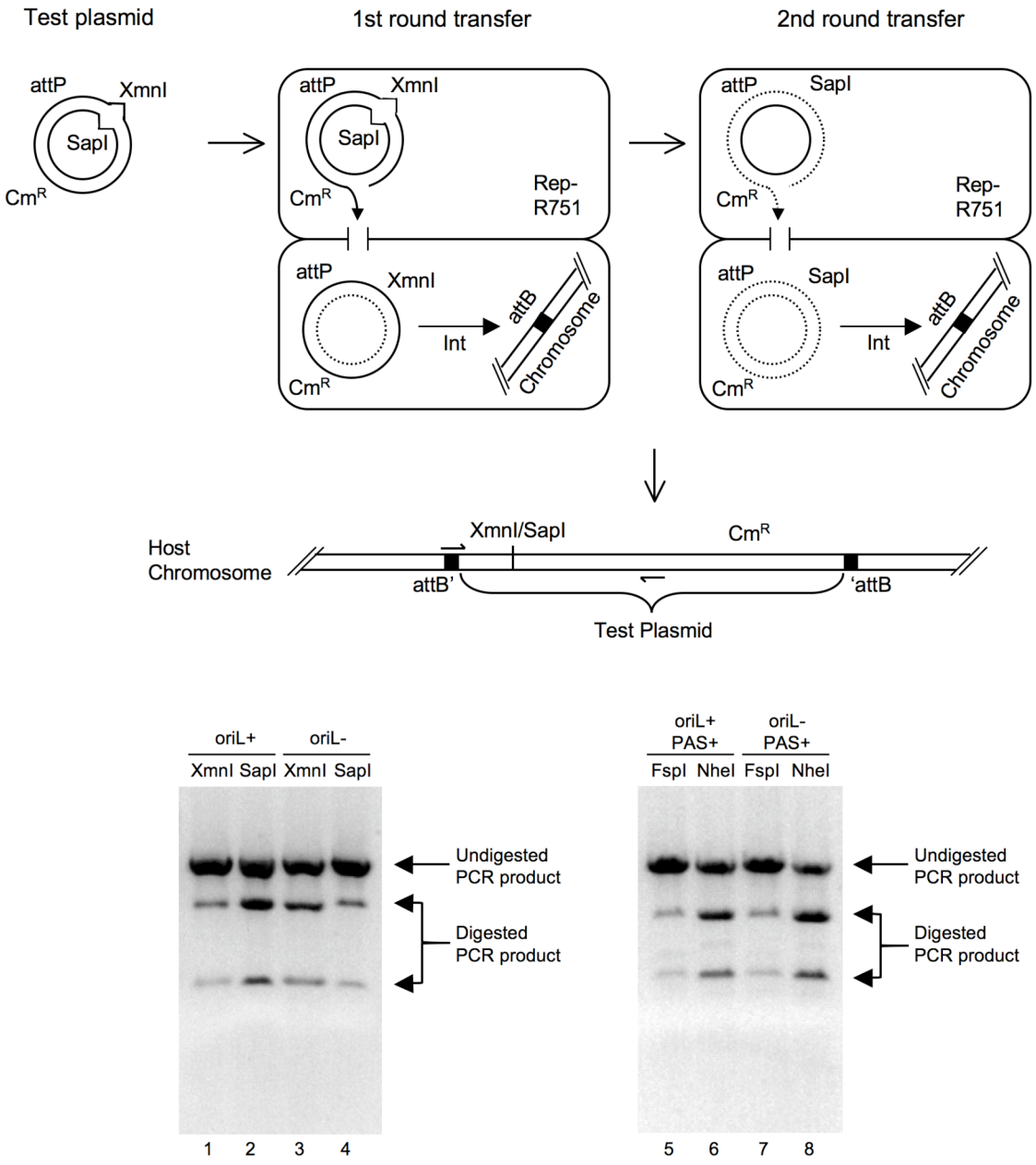


Figure 3.4 – The effect of *oriL* on second round transfer.

Test plasmid, either oriL+ or oriL-, containing an XmnI/SapI mismatch oligo was introduced into donors lacking RepAC. The donor cells were mated with recipient cells containing lambda integrase. Integrated test plasmid was amplified from populations of 25 transconjugants by whole-cell PCR and digested with either XmnI or SapI and analyzed on an agarose gel. The experiment was repeated with an FspI/NheI mismatch oligo in a test plasmid containing a PAS site from pBR322.

E. coli attB: cctgctttttatactaacttga
Salmonella attB: cctgctttttatactaagttga

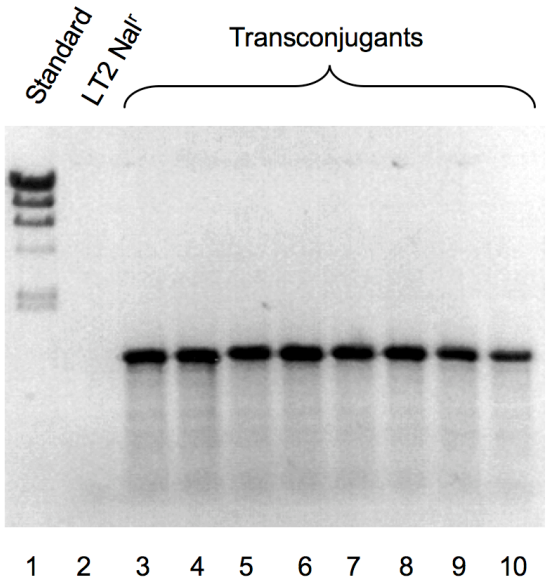
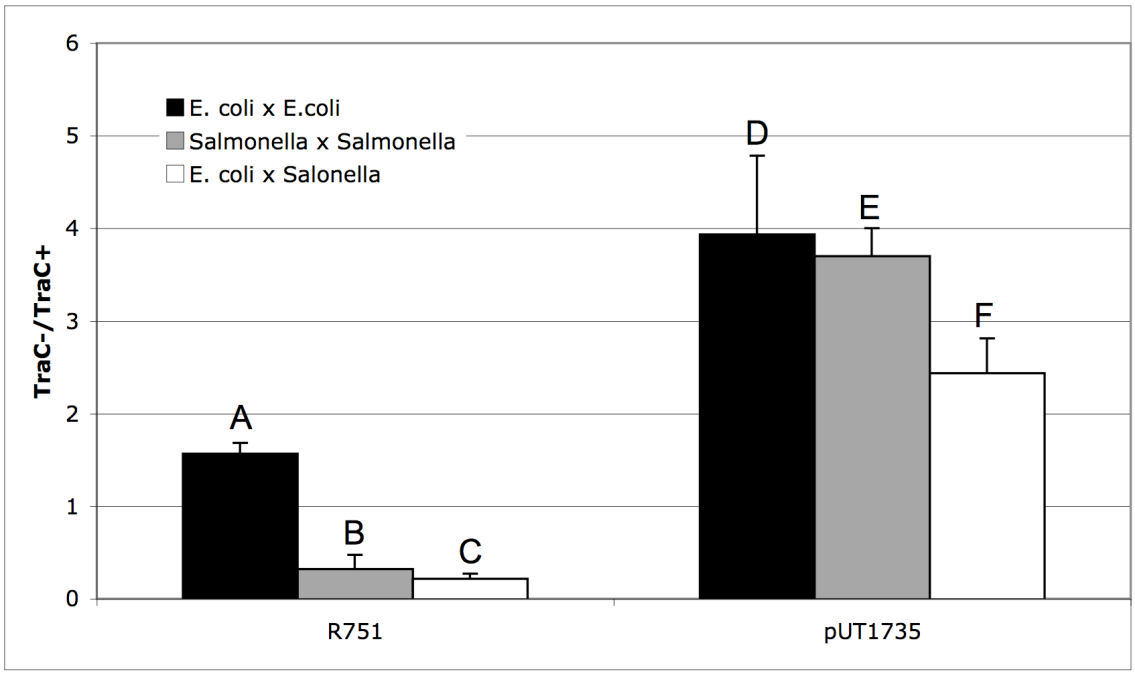


Figure 3.5 – *attP*-containing test plasmids are integrated into *Salmonella* chromosomes.

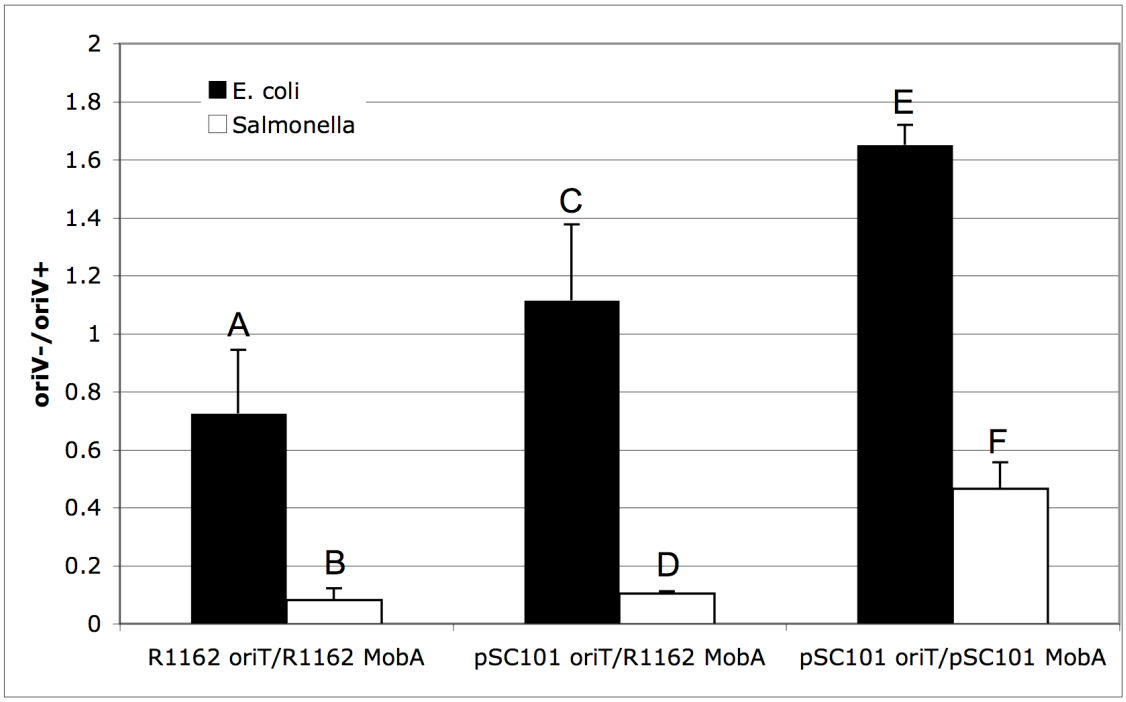
Eight LT2 Nal^r transconjugants were used as template in whole-cell PCR (lanes 3-10). Unmated recipient cells were used as a negative control (lane 2). To ensure that only integrated plasmid DNA was amplified, one primer hybridized to chromosomal DNA while the other hybridized to plasmid DNA.



	Transferred Plasmid	E. coli x E. coli		Salmonella x Salmonella		E. coli x Salmonella	
		TraC+	TraC-	TraC+	TraC-	TraC+	TraC-
1)	R751	0.059	0.092	0.22	0.070	0.071	0.015
2)	pUT1735	0.023	0.087	0.057	0.213	0.042	0.10

Figure 3.6 – Transfer of R751 and pUT1735 in the presence and absence of TraC primase.

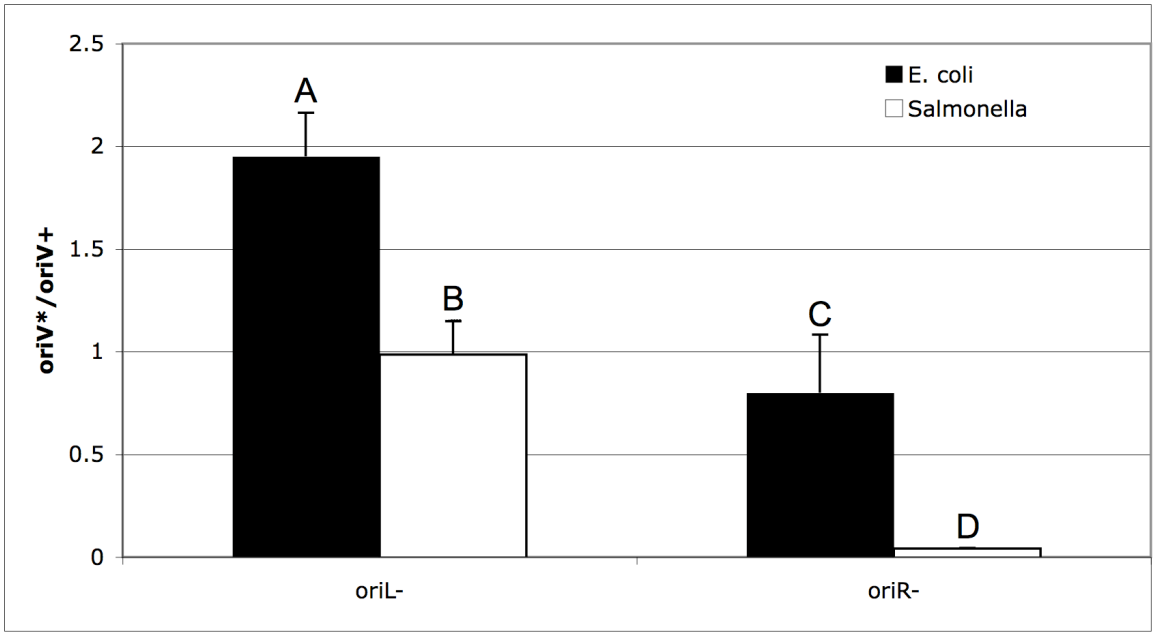
Transfer efficiencies (expressed as transconjugants/donor) for R751 and pUT1735 were calculated after performing intraspecific (*E. coli* x *E. coli* and *Salmonella* x *Salmonella*) and interspecific (*E. coli* x *Salmonella*) matings. Averages from three trials are shown. The graph illustrates the differences of transfer with or without TraC primase.



	Test plasmid	Helper plasmid	E. coli		Salmonella	
			oriV+	oriV-	oriV+	oriV-
1)	R1162 oriT	R1162 MobA	6660	4217	708	46
2)	pSC101 oriT	R1162 MobA	4434	5398	304	32
3)	pSC101 oriT	pSC101 MobA	1227	2028	21	9

Figure 3.7 – Transfer of test plasmids by MobA proteins in *E. coli* or *Salmonella*.

oriV+ or *oriV*- test plasmids containing either R1162 *oriT* or pSC101 *oriT*, where electroporated into donors lacking Rep proteins, but expressing either R1162 MobA or pSC101 MobA. The donor cells were mated with recipients expressing lambda integrase. All matings were intraspecific. The average number of transconjugants/plate is recorded.



	Test plasmid	E. coli	Salmonella
1)	R1162 oriT, oriL+/oriR+	1010	340.75
2)	R1162 oriT, oriL-/oriR+	1997.5	327.75
3)	R1162 oriT, oriL+/oriR-	762.5	14.5

Figure 3.8 – Transfer of test plasmids lacking priming sites in *E. coli* and *Salmonella*.

Test plasmids lacking either *oriL* or *oriR* were electroporated into Rep- donors expressing R1162 MobA. These cells were mated with recipients expressing lambda integrase. The average number of transconjugants/plate is recorded.

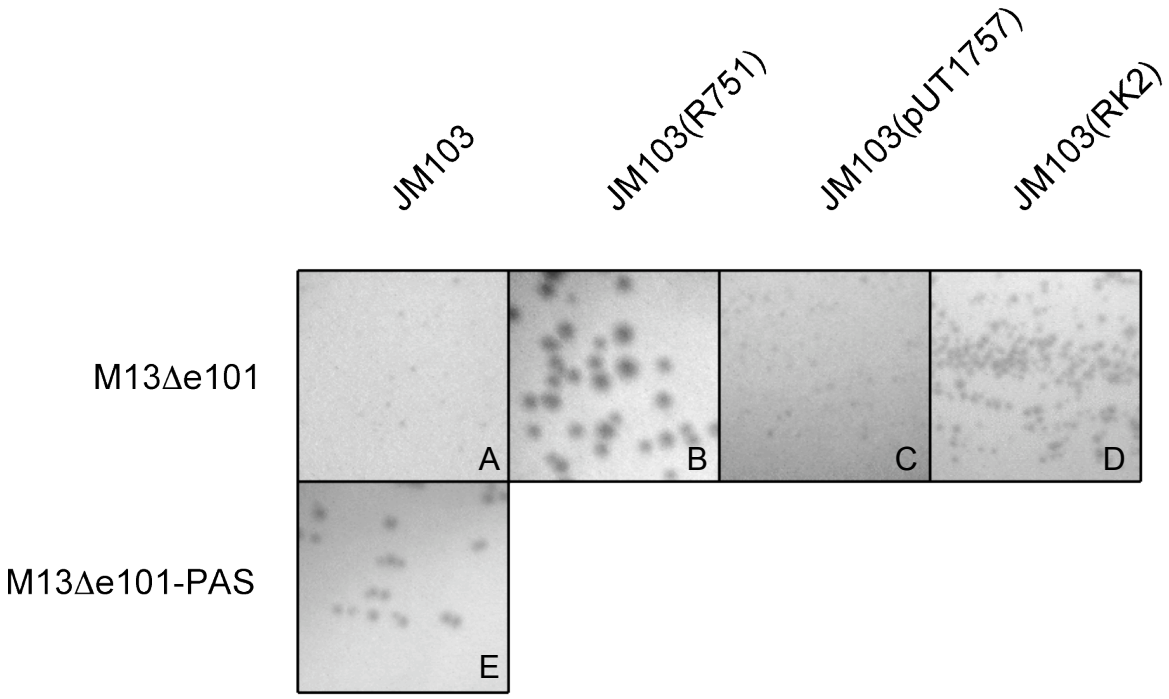


Figure 3.9 – Complementation of origin-deficient phage by R751 primase.

Phage M13Δe101 was introduced into JM103 strains harboring no helper plasmid (A), R751 (B), R751 TraC- (pUT1757) (C) or RK2 (D) and allowed to form plaques on soft agar. As a control, an M13Δe101 phage containing a pBR322 PAS site was also used (E). Representative sections of soft agar plates are shown.

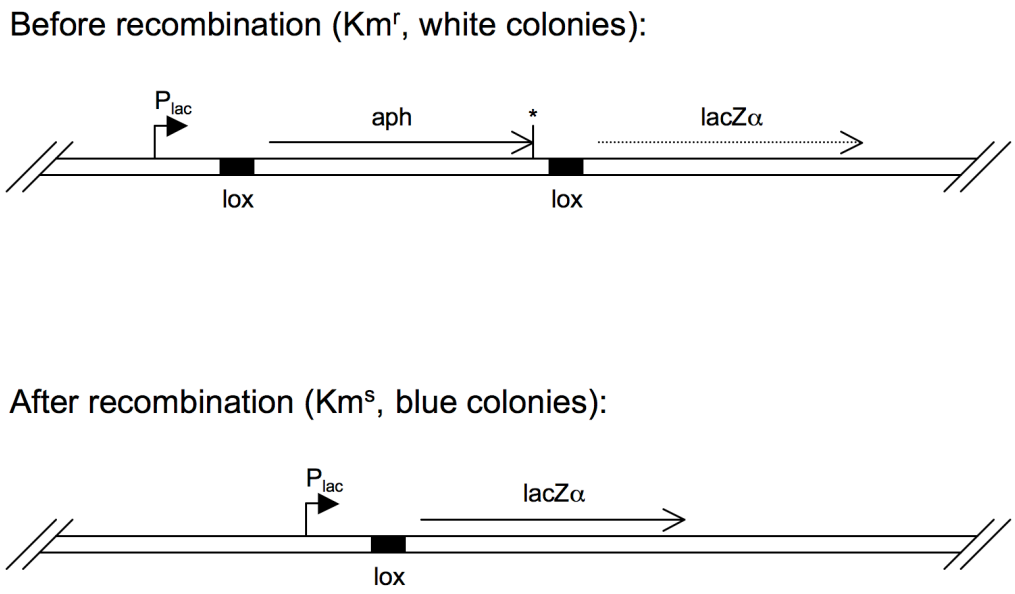


Figure 4.1 – Design of Cre-sensing locus.

Prior to recombination, the gene responsible for kanamycin restance (*aph*) is expressed from the *lac* promoter. Terminators located adjacent to the *aph* ORF (astersisk) prevents translation of the downstream *lacZα* ORF. After recombination by Cre, the *aph* ORF is removed, allowing expression of LacZα.

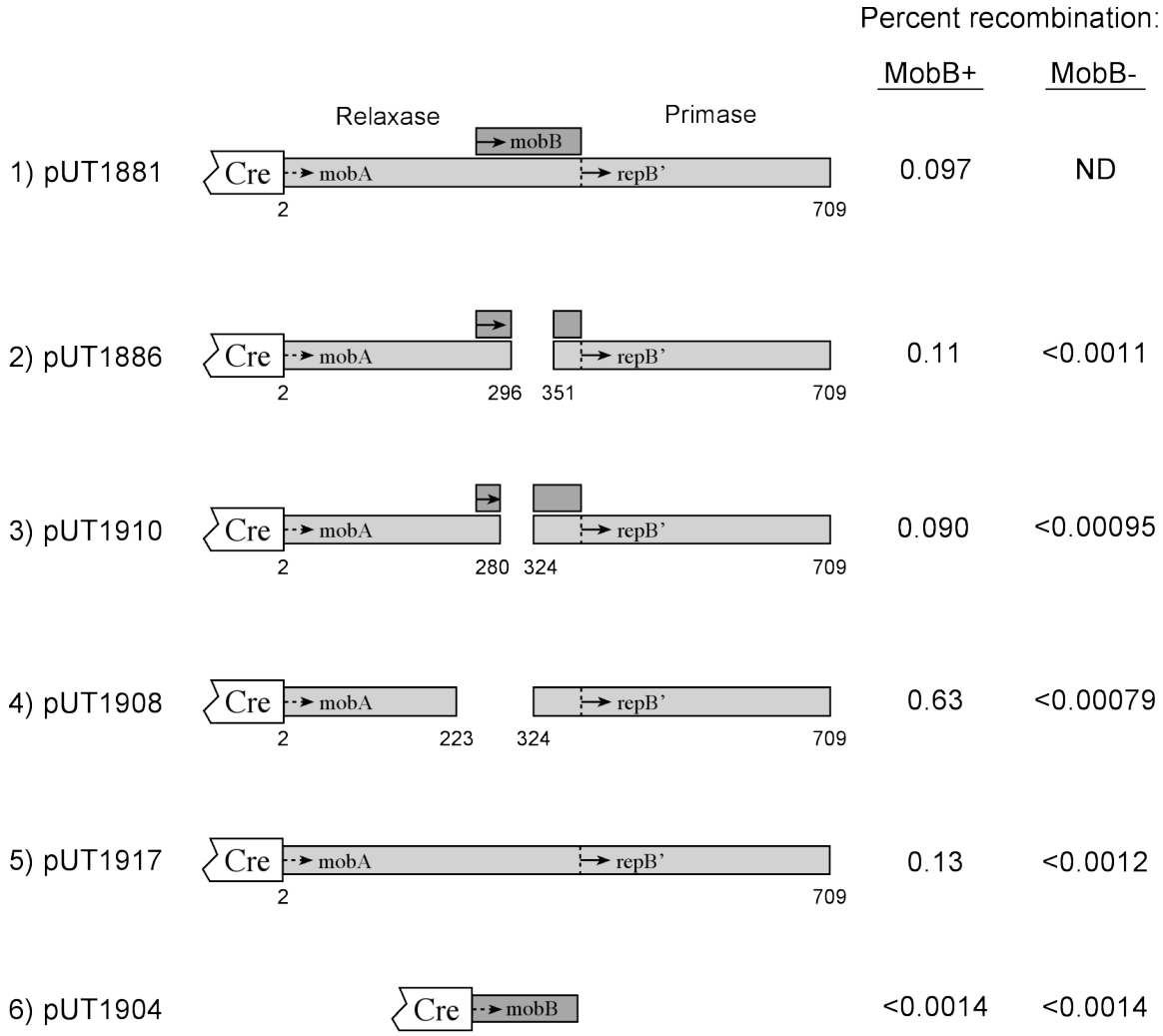


Figure 4.2 – Transfer of MobA derivatives in the presence or absence of MobB.

Full-length MobA or mutants containing internal deletions were fused to the C-terminus of Cre and used in Cre transfer assays. The numbers to the right represent the percent recombination in the presence or absence of MobB. The light gray boxes represent the MobA peptide and the dark gray box represents the MobB peptide. Solid arrows mark the position of start codons while dashed arrow represent start codons that have been removed during the cloning process. The numbers below the peptides refer to residue numbers.

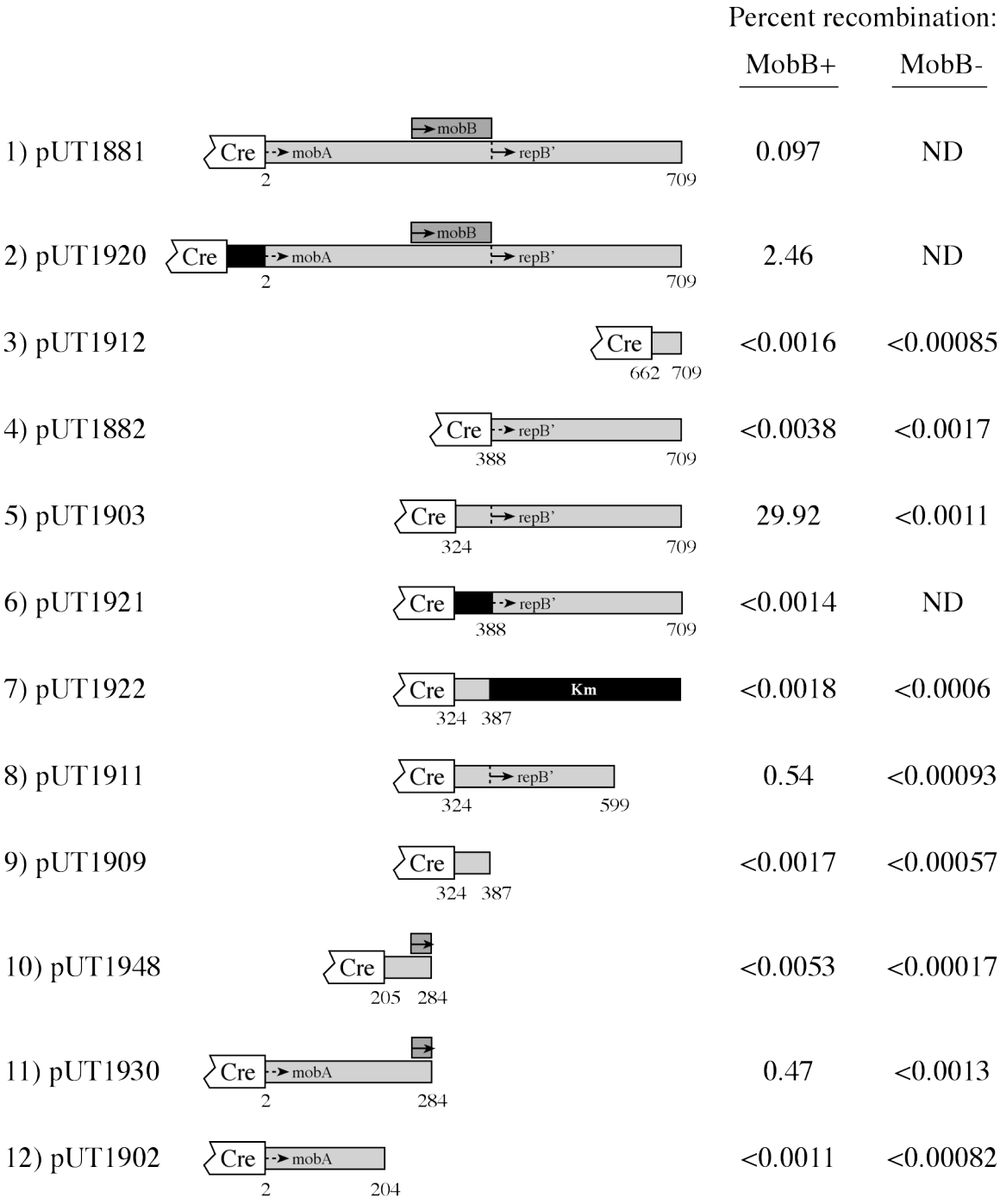


Figure 4.3 – Transfer of the primase and relaxase regions of MobA.

As described in the legend for Figure 4.2. The black boxes in pUT1920 and pUT1921 represent a 64-amino acid fragment of the Aph (kanamycin resistance) peptide.

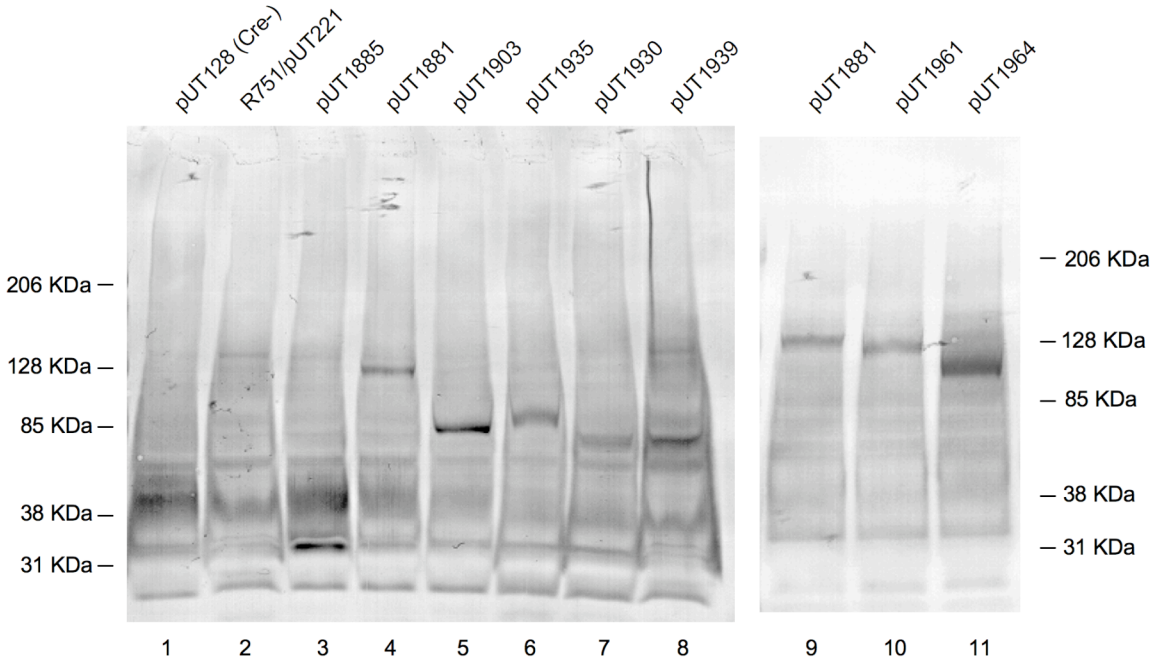


Figure 4.4 – Western blot of select Cre fusions.

Each lane contains 20 μg of lysate from donor cells harboring the indicated plasmid. The blot was probed with anti-Cre antibody.

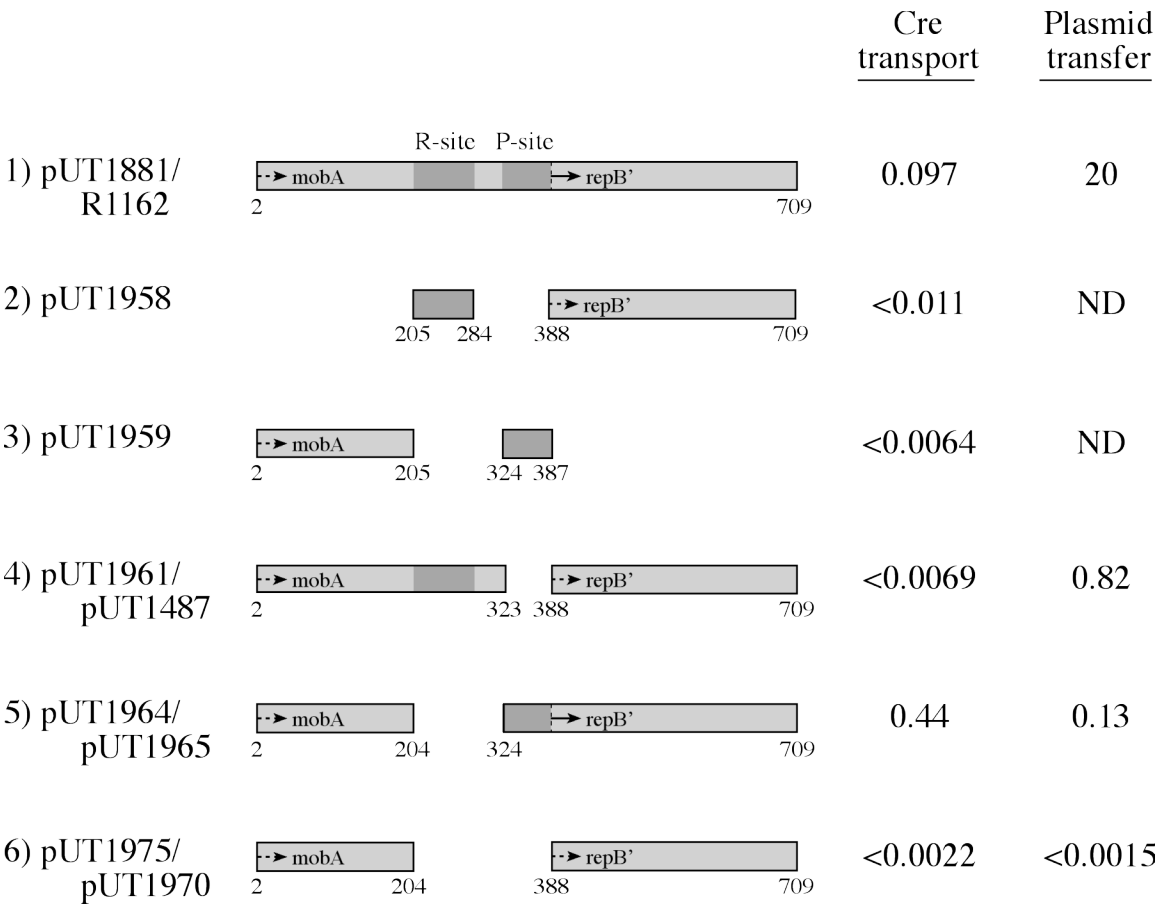


Figure 4.5 – Cre transport and plasmid transfer by MobA molecules with internal deletions.

Cre transport was calculated as described in the materials and methods. Plasmid transfer was calculated as the percentage of R751 transconjugants receiving an R1162-based molecule with the indicated MobA mutation. In cases were two plasmids are listed, the first is the Cre-expression plasmid and the second is the R1162-based plasmid. MobB was supplied by plasmid pUT221 in all donors.

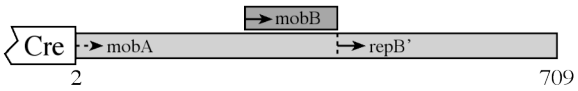
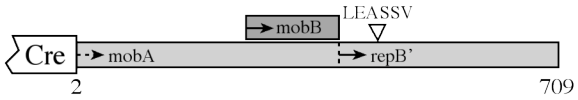
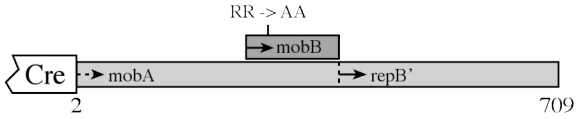
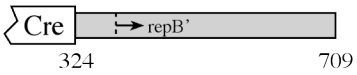
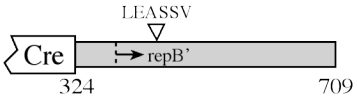
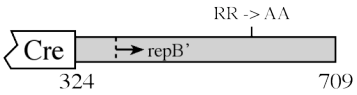
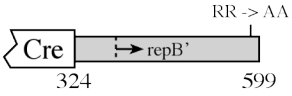
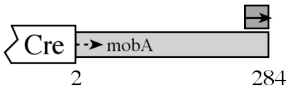
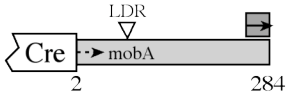
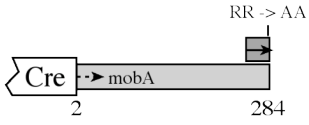
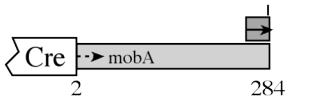
		Percent recombination: MobB+	MobB-
1) pUT1881		0.097	ND
2) pUT1933		<0.0092	ND
3) pUT1966		0.041	ND
4) pUT1903		29.92	<0.0011
5) pUT1935		0.048	<0.0018
6) pUT1938		9.79	<0.00075
7) pUT1942		0.34	<0.00079
8) pUT1930		0.47	<0.0013
9) pUT1947		<0.0036	<0.00071
10) pUT1939		<0.0015	<0.0013
11) pUT1944		<0.0037	<0.00088

Figure 4.6 – Transfer of MobA structural mutants.

As described in the legend for Figure 4.2.

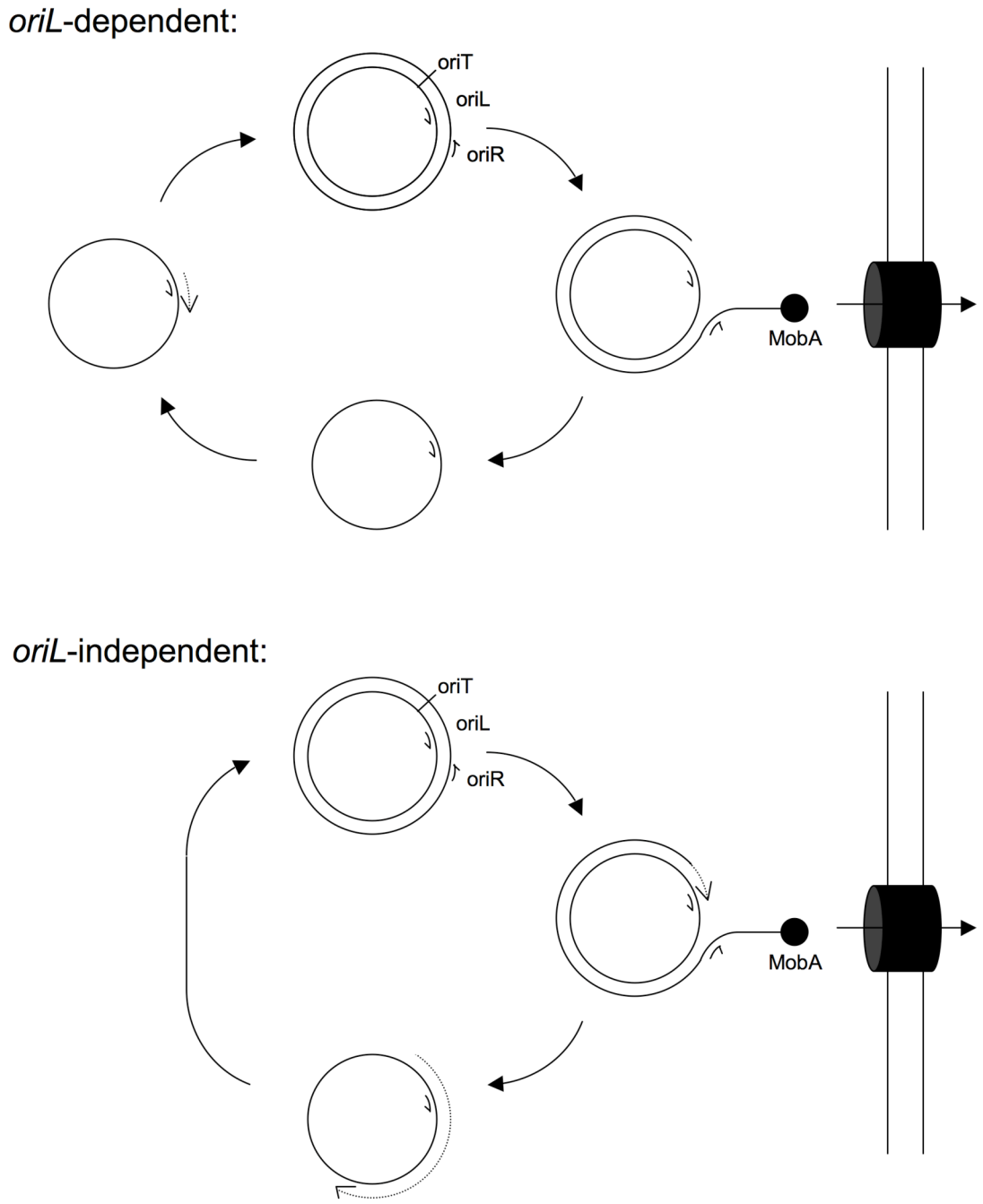


Figure 5.1 – Two models of strand replacement in the donor.

Strand replacement initiated by *oriL* (*oriL*-dependent) or by extension of the exposed 3'-end (*oriL*-independent). Priming sites are represented by half arrows. Nascent DNA is represented by a dotted line.

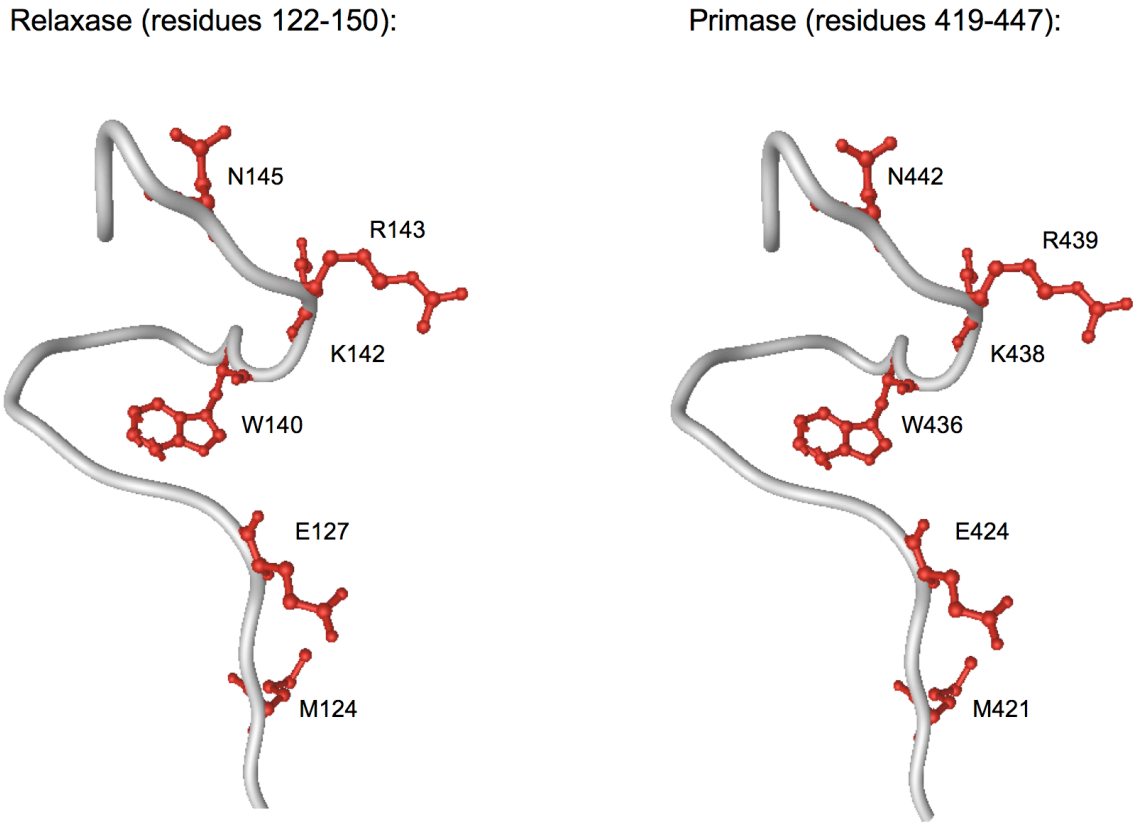


Figure 5.2 – Similarity in structure between relaxase and primase regions in MobA.

The crystal structure of the relaxase portion of MobA was used to perform a structural alignment with the primase portion. Amino acids 122-150 and 419-447 showed the highest degree of structural alignment. Residues common to both regions are labeled.

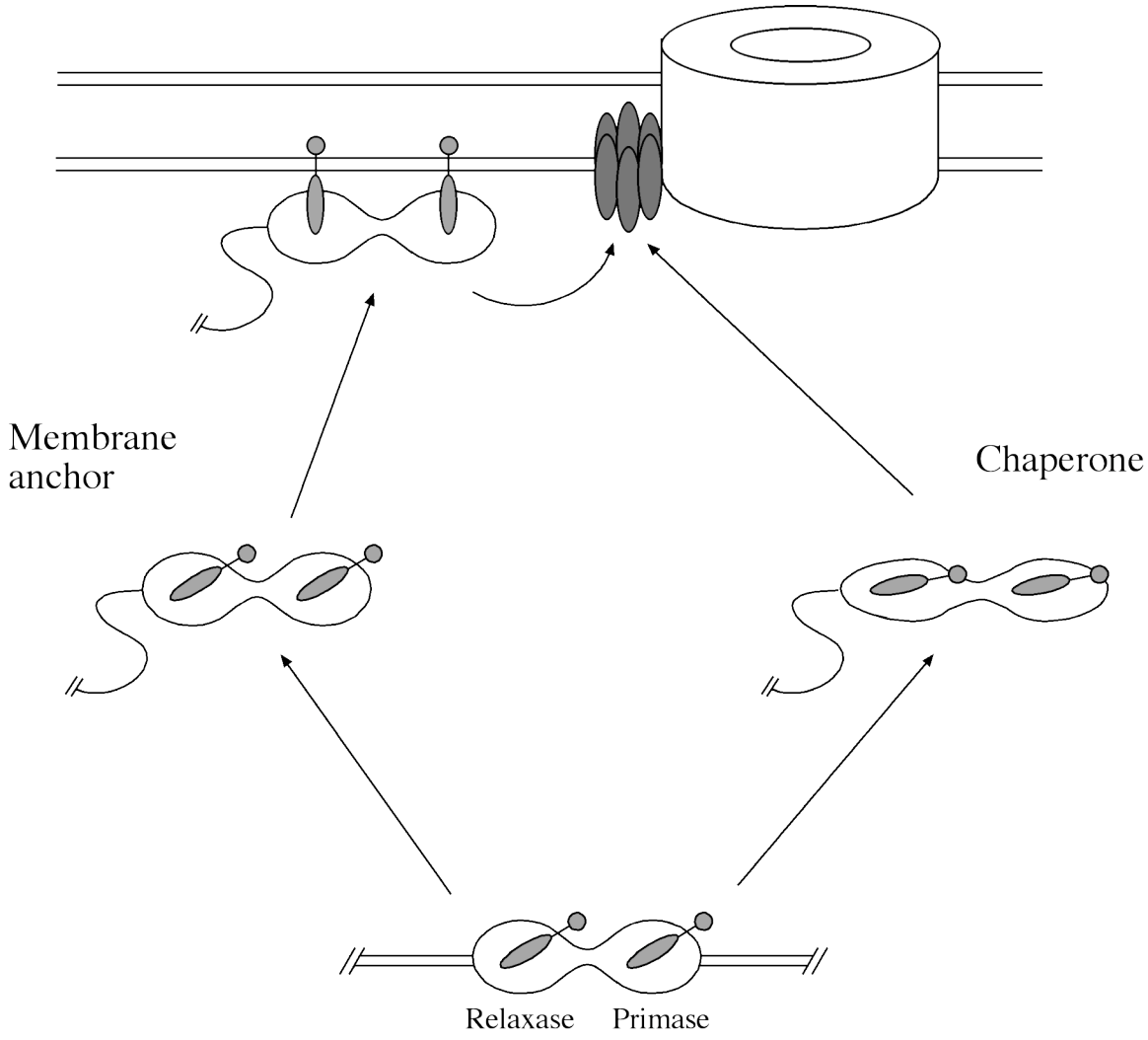


Figure 5.3 – Two models for the role of MobB in MobA transport.

MobB (light grey), interacting with both domains of MobA (white), might act as a membrane anchor or chaperone prior to selection of the MobA-DNA complex by TraG coupling protein (dark grey).

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Vita

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